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INFLUENCE OF VISUAL CUES AND ISOTHIOCYANATE LURES ON CAPTURE OF THE POLLEN BEETLE, *Meligethes aeneus* IN FIELD TRAPS

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Abstract—The effect of trap design, trap color, and isothiocyanate (NCS) lures on the capture of the pollen beetle, *Meligethes aeneus*, was studied in field experiments. Unbaited yellow water and adhesive traps were equally attractive. A small sticky card trap with a yellow upper face, mounted at 45° to the vertical, was attractive throughout the year, but horizontal yellow cards were attractive only in spring, and vertical cards only in autumn. Yellow–green and white water traps were less attractive than yellow, while grass green, cream, and black were unattractive. *M. aeneus* was attracted equally to four alkanyl, three alkenyl, and to 2-phenylethyl isothiocyanate lures, when the release rates were in the range of 5–30 mg/day. Attraction to the most effective unbaited yellow traps was enhanced 1.7–3.3 times with the addition of a lure comprising a mixture of allyl, 3-butenyl, 4-pentenyl, and 2-phenylethyl NCS. Interactions were observed among the visual and odor cues. A yellow sticky card trap, baited with 2-phenylethyl NCS and mounted at 45° to the vertical, may be used throughout the year to monitor *M. aeneus*.

Key Words—*Meligethes aeneus*, pollen beetles, bronzed blossom beetles, Nitidulidae, trap design, trap color, isothiocyanates, attractant.

INTRODUCTION

The genus *Meligethes* (Coleoptera: Nitidulidae: Meligethinae), the bronzed blossom beetles, occurs worldwide and consists of over 400 species (Kirk-Spriggs, 1996). Commonly known as pollen beetles, the adults usually oviposit on a single host plant, although they often feed on a range of families. The brassicaceous *Meligethes* spp. are unusual in that their larvae can complete development in

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several Brassicaceae (= Cruciferae). Of the 10 *Meligethes* spp. reported from brassicaceous plants in Europe, *M. aeneus* is by far the most common on cultivated *Brassica* spp.

On emergence from hibernation, adult *M. aeneus* mature by feeding on pollen of spring flowers of several families (Fritzsche, 1957; Free and Williams, 1978). They then oviposit in brassicaceous buds, preferably those 2–3 mm long (Scherney, 1953; Nilsson, 1988; Ekbom and Borg, 1996). The two larval instars (Osborne, 1964) feed on pollen within the bud and then pupate in the soil. On emergence, the new adults feed on pollen from many families before flying to overwintering sites.

In Europe, *M. aeneus* is an important pest of oilseeds, particularly spring oilseed rape, *Brassica napus*, spring turnip rape, *B. campestris*, brown mustard, *B. juncea*, and white mustard, *Sinapis alba* (Lamb, 1989; Winfield, 1992; Ekbom, 1995). Both adults and larvae contribute to economic loss through destruction of buds and flowers. Within the last 10–20 years, *M. aeneus* populations have increased due to the expansion of the area sown to oilseeds (Hokkanen et al., 1986; Lane and Walters, 1993), and the insect has become a pest of vegetable brassicas (Hokkanen et al., 1986; Finch et al., 1990).

Phytophagous insects, like *M. aeneus*, orient to plants for both feeding and oviposition under the influence of visual and odor stimuli (Miller and Strickler, 1984). Visual and odor cues from rape fields, rather than wind direction, were found to affect the dispersal of *M. aeneus*, which moved up to 9.6 km within 48 hr of being released (Taimr et al., 1967). Several studies have demonstrated attraction to the odor of extracts of brassicaceous plants (Görnitz, 1953; Nolte, 1959; Free and Williams, 1978). In another, rape flower and leaf extracts were attractive in the field from at least 20 m, and it was suggested that at this distance the beetles use odor-mediated upwind anemotaxis to locate oilseed rape plants (Evans and Allen-Williams, 1994). However, Nolte (1959) considered the effect of plant odor on ovipositing females to be secondary to visual attraction and had no effect "over a distance."

In 1926, Wasmann noted that *M. aeneus* is attracted to "rape yellow" color (Wasmann, 1926). Unbaited yellow water traps, such as Moericke pans (Moericke, 1953), were used in Germany in the 1950s to monitor the appearance of the beetle after hibernation (Nolte, 1955; Görnitz, 1956; Fritzsche, 1957). Since then, there have been further reports on attraction of *Meligethes* spp. to colored traps, but in most cases the results have not been statistically analyzed. Usually, yellow traps were the most attractive (e.g., Görnitz, 1956; Fritzsche, 1957; Nolte, 1959; Láska et al., 1986; Buechi, 1990), but in a few circumstances white traps were equally, or more attractive (Fritzsche, 1957; Goos et al., 1976; Košt'ál, 1992). Most trapping of *Meligethes* spp. has been done using water traps, but adhesive ones have been used recently (e.g., Buechi, 1990; Büchs, 1993; Ekbom and Borg, 1993).

Because *M. aeneus* can be trapped readily in unbaited, yellow traps, the insect's response to odor cues, e.g., plant volatile semiochemicals, has been little studied. *Meligethes* spp., like many brassica-feeding insects (Finch and Skinner, 1982; Pivnick et al., 1991, 1992), are attracted to isothiocyanates, which are volatile catabolites of glucosinolates, the thioglycosides typical of the Brassicaceae (Fenwick et al., 1983). Thus, yellow water traps baited with allyl isothiocyanate (NCS) (Finch, 1977; Free and Williams, 1978; Lerin, 1984; Košt'ál, 1992), phenyl NCS (Lerin, 1984), or 3-butenyl, 4-pentenyl, or 2-phenylethyl NCS (Smart et al., 1993, 1995) were more attractive than unbaited yellow traps.

In this paper, we describe field-trapping experiments in which we examined the effects of trap design, color, and isothiocyanate lures on capture of *M. aeneus*. The relationship between responses to colored traps and the spectral reflectances of the traps was studied, and the interaction between visual and olfactory cues was investigated. The responses were compared with those of another rapeseed pest, the cabbage seed weevil, *Ceutorhynchus assimilis*.

METHODS AND MATERIALS

Experimental Design and Data Analysis. Nine field experiments were conducted on Rothamsted Farm, Harpenden, U.K., during 1991–1994, and were part of a continuing series. Each experiment comprised the randomized block (Latin square) design described previously (Smart and Blight, 1997; Smart et al., 1997). Traps representing one replicate (i.e., row) of a block were set out in a straight line at 10-m spacing. Captured insects were removed at regular intervals and were identified and counted in the laboratory. Blocks were usually rerandomized when a mean of at least 10 beetles per treatment had been captured in a replicate.

Total trap catch data were transformed by $\log_{10}(x + 1)$, and analyses of variance (ANOVA) were performed. Where appropriate, transformed treatment means were compared at $P = 0.05$ by using Duncan's multiple range test (Duncan, 1955). Means were then transformed back, and these are shown in the tables.

Water Bowl Traps. These were used for all field studies except experiments 1 and 2, and have been described previously (Smart et al., 1997). They were brush painted and were two thirds filled with an aqueous detergent solution (0.5%). Colors used were canary yellow (ICI autocolour BS381/cP383, BS0409) (for all experiments, except 1 and 2); brilliant white (BS0101) (experiments 3–5); black (BS0122) (experiment 3); grass green (BS0024) (experiments 3–5); deep cream (BS0453) (experiments 5 and 6); and yellow–green (experiments 5 and 6). The latter color was a 50 : 50 mixture of canary yellow and grass green. Lures were wired to a clear plastic cross-bar, secured across the top of the bowl.

Petri Dish Water Traps. Petri dish water traps (Smart et al., 1997) were used in experiment 1. They were painted canary yellow and filled with 0.5% aqueous detergent solution. Lures were wired above the trap to a piece of plastic pipe glued to the center of the inside of the dish.

Yellow Sticky Card Traps. These traps were coated with Oecotak A5 (Oecos Ltd., Kimpton, Herts, U.K.) and were used in experiments 1 and 2. They were mounted horizontally, vertically, or at 45° to the vertical. Each sticky card was clipped to an equal-sized piece of grey plastic sheet mounted on a pole. The yellow face of the horizontal trap pointed upwards, and those of the 45°-angled and vertical traps pointed towards the field. Lures were held in position above the traps with wire (Smart et al., 1997). All traps were mounted on stakes or poles, approximately 1 m above ground level.

Spectral Reflectance Measurements. The spectral reflectances of the colored bowl traps and the yellow sticky card were measured in a darkened room with a Bentham spectrophotometer (model DM150EC). Reflectance was recorded at 5-nm intervals from 350 to 700 nm by using white PTFE as the reference standard.

Chemicals. Nonane (99%), allyl NCS (94%), butyl NCS (99%), ethyl NCS (97%), and 2-phenylethyl NCS (99%), were obtained from Aldrich Chemical Co. Pentyl NCS and propyl NCS (both 97%) were obtained from Avocado Research Chemicals Ltd., Lancashire, U.K. 3-Butenyl NCS (99%) and 4-pentenyl NCS (97%) were synthesized (Dawson et al., 1993).

Lures. Wick dispenser type 1 (Smart et al., 1997) was used in experiment 1 to release a mixture of allyl, 2-phenylethyl, 3-butenyl, and 4-pentenyl NCS in nonane. Release rates at 20°C were ca. 60 mg/day for allyl NCS and ca. 6 mg/day for the other three isothiocyanates.

Wick dispenser type 2 (Smart et al., 1997) was used in experiment 9 to release allyl NCS at 470, 940, and 1880 mg/day.

Polyethylene bag dispensers were used in all other experiments. Each compound was applied to a piece of cellulose sponge (3 mm thick, Code 0032 6865, or 10 mm thick, Code 0013 4811, J. Sainsbury plc) that was heat-sealed into a bag made from polyethylene tubing (Al Packagings Ltd., London). Separate bags were used for each compound and different release rates were obtained by altering the type and surface area of the sponge and the gauge of the polyethylene. Release rates are shown in tables.

Field Experiments. These were: experiments 1 and 2—response to yellow sticky card traps inclined at different angles, experiments 3–6—effect of color on response to bowl water traps, and experiments 7–9—responses to single isothiocyanate lures. In experiments 1–4, the traps were both unbaited and baited with a mixture of allyl, 3-butenyl, 4-pentenyl, and 2-phenylethyl NCS in order that trap type × lure (experiments 1 and 2) and color × lure (experiments 3 and 4) interactions could be examined.

RESULTS

Experiments 1 and 2 (Table 1): Response to Yellow Sticky Card Traps Inclined at Different Angles. Traps were mounted vertically, horizontally, and at 45° to the vertical. Experiment 1 was done in late summer when the new generation of *M. aeneus* was feeding before hibernation, and experiment 2 in the spring when overwintered beetles had become established on the crop.

Factorial ANOVA showed that the angle of inclination of the trap had a significant effect on the numbers of beetles trapped ($P < 0.001$ for trap effect, in both experiments). The trap inclined at 45° caught the most beetles in both experiments, but there were differences between the responses to the vertical and horizontal traps. The new generation of beetles (experiment 1) were equally responsive to the 45°-angled and vertical traps but were less responsive to the

TABLE 1. *Meligethes aeneus* CAUGHT ON YELLOW STICKY CARD TRAPS

Trap	*M. aeneus* caught per replicate (mean)[a]	
	Nonane-baited	NCS-baited[b]
Experiment 1: August 13–September 5, 1991[c]		
Sticky card, 45° to vertical	72.8 ab	122.0 a
Sticky card, vertical	47.4 bc	85.1 ab
Sticky card, horizontal	9.7 e	23.9 d
Petri dish water trap	21.1 d	28.1 cd
	Unbaited	
Experiment 2: April 21–June 23, 1992[d]		
Sticky card, 45° to vertical	40.1 bc	87.3 a
Sticky card, vertical	3.9 d	24.2 c
Sticky card, horizontal	42.4 b	73.5 a

[a]Within each experiment, means followed by different letters are significantly different (Duncan's multiple range test, $P = 0.05$; eight means were compared in experiment 1 and six in experiment 2.

[b]The lure was a mixture of allyl, 3-butenyl, 4-pentenyl, and 2-phenylethyl isothiocyanate (NCS). Allyl NCS was released at ca. 60 mg/day, and each of the other NCS at ca. 6 mg/day.

[c]4594 *M. aeneus* were caught in eight replicates. Factorial ANOVA gave for trap effect: $F = 29.04$; $df = 3, 42$; $P < 0.001$; for lure effect: $F = 13.33$; $df = 1, 42$; $P < 0.001$; for lure × trap interaction: $F = 0.60$; $df = 3, 42$; $P = 0.617$. SE of a treatment mean was 0.09.

[d]5301 *M. aeneus* were caught in two blocks (12 replicates) done consecutively on the same site. Factorial ANOVA gave for trap effect: $F = 57.68$; $df = 2, 43$; $P < 0.001$; for lure effect: $F = 45.34$; $df = 1, 43$; $P < 0.001$; for lure × trap interaction: $F = 5.24$; $df = 2, 43$; $P = 0.009$. SE of a treatment mean was 0.08.

horizontal trap. In contrast, overwintered beetles (experiment 2) were attracted equally to the 45°-angled and horizontal traps but were less responsive to the vertical trap.

In experiment 1, the Petri dish water trap with its small vertical edge was slightly more attractive than the horizontal trap, but less attractive than the other two traps. In both experiments, addition of the isothiocyanate lure increased catch significantly ($P < 0.001$ for lure effect in both). In experiment 1, the lure × trap type interaction was not significant ($P = 0.617$) but it was in experiment 2 ($P = 0.009$).

Experiments 3–6 (Table 2): Effect of Color on Response to Bowl Water Traps. The effect of five different colors or shades on capture in unbaited traps was examined, and the influence of an isothiocyanate lure on response to color also was investigated. Unbaited canary yellow traps were the most attractive and captured at least 71% of the total beetles caught in each experiment. There was some attraction to yellow–green, which caught 17.5% of the total number in experiment 5, but responses to white and grass green traps were lower and variable. Both the black and cream traps were unattractive.

Spectral reflectances of the colored traps are shown in Figure 1. The most attractive color, canary yellow, had a high degree of unsaturation (reflected over wide spectral range) and had >40% reflectance between 515 and 700 nm when compared to the reference standard. The most saturated (spectrally pure) colors were yellow–green (peak at 540–550 nm) and grass green (peak at 525–540 nm), but the peak reflectances were weaker (ca. 40% and 20%, respectively) than that of canary yellow (>80%, 565–700 nm region). The cream shade had a reflectance pattern similar to canary yellow between 510 and 700 nm, but had >20% reflectance in the region 400–470 nm where the canary yellow reflectance was <5%. White was highly unsaturated with >40% reflectance from 400 to 700 nm, while black had <5% reflectance throughout the 350- to 700-nm range. The yellow Oecos sticky card trap used in experiments 1 and 2 was less bright (had lower intensity) than the canary yellow bowl (Figure 1), but otherwise the reflectance spectra of the two were similar.

The addition of a mixed isothiocyanate lure to the colored bowls affected beetle capture. In the two factorial experiments (3 and 4), the lure effect was significant ($P < 0.001$ in both). Canary yellow, grass green, and black baited traps were more attractive than the corresponding unbaited traps. White baited traps did not catch more beetles than the corresponding unbaited traps. The lure × color interaction effect was not significant in experiment 3 ($P = 0.178$), but it was in experiment 4 ($P = 0.048$).

Experiments 5 and 6 had the same experimental design but traps were unbaited in experiment 5 and baited with the mixed isothiocyanate lure in experiment 6. The effect of the bait could not be examined statistically, but inspection of the proportional (%) responses to the various colors suggests that addition

TABLE 2. RESPONSE OF *Meligethes aeneus* TO COLORED BOWL WATER TRAPS

	M. aeneus caught per replicate			
	Unbaited		NCS-baited[a]	
Trap color	Mean[b]	%[c]	Mean[b]	%[c]
Experiment 3[d]				
Canary yellow	121.5 b	84.1	266.3 a	82.9
White	21.0 cd	14.6	40.1 c	12.5
Grass green	1.6 f	1.1	10.5 d	3.2
Black	0.3 f	0.2	4.4 e	1.4
Experiment 4[e]				
Canary yellow	23.4 b	84.8	78.1 a	85.1
White	3.7 c	13.4	6.5 c	7.1
Grass green	0.5 d	1.8	7.2 c	7.8
	Experiment 5[f]		Experiment 6[g]	
Canary yellow	269.4 a	71.7	682.8 a	58.6
Yellow–green	65.5 b	17.5	189.5 b	16.3
Grass green	18.5 c	4.9	122.8 c	10.5
White	16.6 c	4.4	81.9 d	7.0
Cream	5.6 d	1.5	89.1 cd	7.6

[a]The lure was a mixture of allyl, 3-butenyl, 4-pentenyl, and 2-phenylethyl isothiocyanates (NCS). In experiment 3, allyl NCS was released at ca. 60 mg/day, and each of the other NCS at ca. 6 mg/day. In experiments 4 and 6, allyl NCS was released at ca. 25 mg/day and 3-butenyl, 4-pentenyl and 2-phenylethyl NCS at ca. 5 mg/day.

[b]Within each experiment, means followed by different letters are significantly different (Duncan's multiple range test, $P = 0.05$. Means compared: experiment 3, eight; experiment 4, six; experiment 5, five; experiment 6, five).

[c]Percentage of total number of beetles caught in either unbaited or baited traps.

[d]4643 *M. aeneus* were caught in eight replicates, June 1–July 15, 1992. Factorial ANOVA gave for color effect: $F = 128.53$; $df = 3, 42$; $P < 0.001$; for lure effect: $F = 41.77$; $df = 1, 42$; $P < 0.001$; for color × lure interaction: $F = 1.72$; $df = 3, 42$; $P = 0.178$. SE of a treatment mean was 0.10.

[e]905 *M. aeneus* were caught in six replicates, June 21–July 6, 1993. Factorial ANOVA gave for color effect: $F = 68.07$; $df = 2, 20$; $P < 0.001$; for lure effect: $F = 35.45$; $df = 1, 20$; $P < 0.001$; for color × lure interaction: $F = 3.54$; $df = 2, 20$; $P = 0.048$. SE of a treatment mean was 0.10.

[f]2147 *M. aeneus* were caught in five replicates, May 1–June 2, 1994. ANOVA gave for treatment effect: $F = 90.61$; $df = 4, 24$; $P < 0.001$. SE of a mean was 0.07.

[g]6996 *M. aeneus* were caught in five replicates, May 1–June 2, 1994. ANOVA gave for treatment effect: $F = 55.33$; $df = 4, 24$; $P < 0.001$. SE of a mean was 0.05.

of the lure had an effect on the proportions of beetles caught by the different colors.

Experiments 7–9 (Tables 3–5): Responses to Single Isothiocyanate Lures. In experiment 7 (Table 3), the responses to yellow water bowl traps baited with individual alkanyl and alkenyl isothiocyanate lures were compared to the response to an unbaited trap and to isothiocyanate multilures. All isothiocyanate

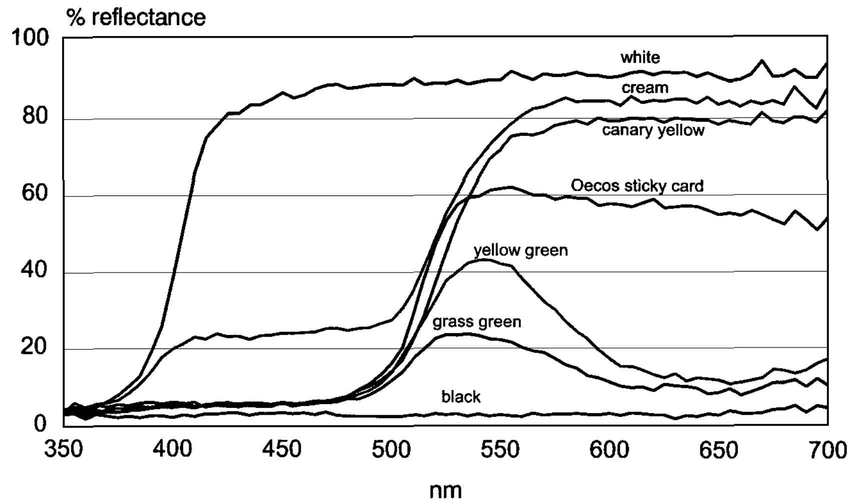

FIG. 1. Spectral reflectance curves for the six colored water traps and the Oecos sticky card trap.

lures were more attractive than the unbaited trap. There were no differences in attraction among the single isothiocyanate lures. The alkanyl and alkenyl NCS multilures, both of which also contained 2-phenylethyl NCS, were equally attractive and more attractive than the individual isothiocyanates (Duncan's multiple range test, $P = 0.05$).

Experiment 8 (Table 4) examined the effect of including 2-phenylethyl NCS in the alkanyl and alkenyl isothiocyanate lures. The multilures, with or without 2-phenylethyl isothiocyanate, were equally attractive. The trap baited only with 2-phenylethyl NCS caught more beetles than the unbaited trap but fewer than the isothiocyanate multilures (Duncan's multiple range test, $P = 0.05$).

Experiment 9 (Table 5) examined the response of *M. aeneus* to yellow water bowl traps baited with lures releasing large amounts (470–1880 mg/day) of allyl NCS. All lures caught more beetles than the nonane-baited and unbaited traps, which were equally unattractive. Release of 470 mg/day of allyl NCS attracted fewer beetles than release of 1880 or 940 mg/day (Duncan's multiple range test $P = 0.05$).

DISCUSSION

A proportion of pollen beetles captured during this study was identified to species, and all were *M. aeneus* (Smart, unpublished). No *M. viridescens*

TABLE 3. EXPERIMENT 7: RESPONSE OF *Meligethes aeneus* TO YELLOW WATER TRAPS BAITED WITH ALKANYL AND ALKENYL ISOTHIOCYANATES (NCS)

Lure	Nominal release rate (mg/day)	*M. aeneus* caught per replicate (mean)[a]
Unbaited		18.5 c
Propyl NCS	25	80.8 b
Butyl NCS	12	67.6 b
Pentyl NCS	11	68.7 b
Allyl NCS (2-propenyl NCS)	30	71.3 b
3-Butenyl NCS	5	84.1 b
4-Pentenyl NCS	5	85.5 b
Alkenyl NCS[b] + 2-phenyl-ethyl NCS[c]	45	112.5 a
Alkanyl NCS[d] + 2-phenyl-ethyl NCS[c]	53	121.9 a

[a]8855 *M. aeneus* were caught in nine replicates, August 10–18, 1993. ANOVA gave $F = 32.61$; $df = 8, 56$; $P < 0.001$. Means followed by different letters are significantly different (Duncan's multiple range test, $P = 0.05$). SE of a treatment mean was 0.04.
[b]Allyl, 3-butenyl, and 4-pentenyl NCS were released separately at the rates shown above.
[c]2-Phenylethyl NCS was released at ca. 5 mg/day.
[d]Propyl, butyl, and pentyl NCS were released separately at the rates shown above.

TABLE 4. EXPERIMENT 8: EFFECT ON CAPTURE OF *Meligethes aeneus* OF 2-PHENYLETHYL NCS IN ISOTHIOCYANATE (NCS) MULTIBAITS

Lure	Nominal release rate (mg/day)	*M. aeneus* caught per replicate (mean)[a]
Unbaited		48.6 c
2-Phenylethyl NCS	5	120.6 b
Alkenyl NCS[b] + 2-phenyl-ethyl NCS[c]	45	172.8 a
Alkanyl NCS[d] + 2-phenyl-ethyl NCS[c]	40	188.2 a
Alkenyl NCS[b]	40	158.2 ab
Alkanyl NCS[d]	35	207.0 a

[a]15,133 *M. aeneus* were caught in two blocks (12 replicates), on different sites, April 25–May 12, 1994. ANOVA gave $F = 22.85$; $df = 5, 40$; $P < 0.001$. Means followed by different letters are significantly different (Duncan's multiple range test, $P = 0.05$). SE of a treatment mean was 0.05.
[b]Allyl NCS (release rate ca. 30 mg/day), 3-butenyl NCS (ca. 5 mg/day), 4 pentenyl NCS (ca. 5 mg/day). Each NCS was released separately.
[c]2-Phenylethyl NCS was released at ca. 5 mg/day.
[d]Propyl NCS (ca. 25 mg/day), butyl NCS (ca. 5 mg/day), pentyl NCS (ca. 5 mg/day). Each NCS was released separately.

TABLE 5. EXPERIMENT 9: RESPONSE OF *Meligethes aeneus* TO YELLOW WATER TRAPS BAITED WITH LARGE AMOUNTS OF ALLYL ISOTHIOCYANATE (NCS)

Lure	Nominal release rate (mg/day)	*M. aeneus* caught per replicate (mean)[a]
Nonane		16.4 c
Allyl NCS		
100%	1880	44.8 a
50% in nonane	940	41.0 a
25% in nonane	470	28.8 b
Unbaited		14.8 c

[a]1817 *M. aeneus* were caught in two blocks (10 replicates), on different sites, June 12–August 10, 1992. ANOVA gave $F = 21.52$; $df = 4, 24$; $P < 0.001$. Means followed by different letters are significantly different (Duncan's multiple range test, $P = 0.05$). SE of a treatment mean was 0.05.

were found because its usual host plant, spring rape, was absent from the area (Fritzsche, 1957).

There is little published information on the effect of trap design on capture of *Meligethes* spp. In general, unbaited yellow water traps have been used in Europe to monitor flight activity and/or establish economic thresholds (Lechapt, 1980; Berger, 1987; Košt'ál, 1992; Šedivý and Kocourek, 1994). This is surprising since adhesive traps are generally more practical to use for detection and monitoring and have been used successfully with other insect species (Muirhead-Thomson, 1991).

In preliminary experiments, we examined the effectiveness of a variety of yellow traps, including a delta trap (Oecos Ltd.), a boll weevil Scout cone trap (Forey and Quisumbing, 1987), a funnel trap similar to the Lindgren (Lindgren, 1983), and a BrassicEye trap (used to monitor the cabbage root fly) (Linton, 1998). All caught negligible numbers of beetles, but a sticky box and Petri dish and bowl water traps were equally effective (Smart et al., 1993). In the present work, the small sticky card mounted at an optimum angle was more effective than the Petri dish water trap. Our results contrast with reports that yellow sticky traps are less effective than yellow water traps (Hokkanen et al., 1986; Košt'ál, 1992). This may relate to the traps' reflectance.

Newly emerged and overwintered beetles responded differently to traps mounted at different angles. Like the seed weevil, *C. assimilis* (Smart et al., 1997), new generation *M. aeneus* emerging from the soil in the summer were not captured on horizontal traps. The beetles were attracted, however, to the (horizontal) Petri dish trap, which had a vertical yellow edge and yellow underside. Overwintered beetles in flight in the spring detected the horizontal and 45° angle traps. Thus, we concluded that 45° angle traps could be used throughout the year for detection and monitoring of both species.

We studied the effects of trap color and isothiocyanate lures on beetle capture with water traps because these are the most convenient for research. As with *C. assimilis* (Smart et al., 1997), and in agreement with most published information relating to *Meligethes* spp., the canary yellow trap was consistently the most attractive. In hue, it was similar to *B. napus* flowers (Wäckers, 1994), although it did not reflect in the near UV (350–400 nm) as the flowers do. Since *Meligethes* spp. are flower foragers and pollen feeders, they, like other flower-inhabiting insects, have an innate attraction to yellow (Wäckers, 1994). Although the beetles orient to brassicaceous hosts at the bud stage, populations were observed to be low until yellow buds were present (Winfield, 1961; Buechi, 1990).

A variety of factors appear to have affected the response to the other colored, unbaited traps. Presumably yellow–green was less attractive than canary yellow because its reflectance intensity (from 510 to 700 nm) was much lower. Its hue, though, was similar to that of green leaves (see Kennedy et al., 1961; Gates, 1980; Wäckers, 1994), as was that of the grass green shade. The latter was less attractive, possibly because its intensity (brightness) was low. Black was unattractive because it did not reflect. When various sections of yellow water traps were painted black, the catches were reduced (Finch, 1991).

Responses to the white and cream traps were unexpectedly low considering their intense reflection in the 520- to 700-nm spectral region. This may have been due to reflection in the blue spectral region (400–500 nm) which was either intense (white trap) or weak (cream). "Light blue to dark green" has been said to be repellent to *Meligethes* spp. (Nolte, 1959). Since the degree of attraction to a color can be governed by interactions between key wavelengths (Vernon and Gillespie, 1990), trap reflection in the 400- to 500-nm region possibly inhibited attraction to the 520- to 700-nm region. The relationship between degree of inhibition and reflection intensity is unclear—the weaker 400–500 nm reflection of the cream trap had the greater effect.

The white and cream traps may also have been relatively ineffective because they did not reflect in the near UV (350–400 nm). *Meligethes* spp. perceive white flowers largely through reflection of UV light (Nolte, 1959), and white repels when the amount of UV reflected is either great or small. The white traps, which were as attractive as yellow (Fritzsche, 1957; Goos et al., 1976; Košt'ál, 1992), may have reflected in the UV. However, variable responses to color can also result from variations in the physiological state of the responding insects (Kirk, 1984; Scherer and Kolb, 1987; Wäckers, 1994) or to background effects (Nolte, 1959; Košt'ál and Finch, 1996). In at least one instance, unbaited blue traps were as attractive as yellow (Ekbom and Borg, 1993).

Previous work has shown that *Meligethes* spp. are attracted in the field to 2-phenylethyl and phenyl NCS and to three alkenyl isothiocyanates. At comparable release rates, 5–30 mg/day, the corresponding alkanyl (propyl, butyl, and pentyl) analogs are similarly attractive, as is ethyl NCS (Smart and Blight,

unpublished). Mixtures of the alkanyl and alkenyl isothiocyanates with or without 2-phenylethyl NCS, are generally more attractive than the individual isothiocyanates, but this may be ascribed to the higher total release rates (35–53 mg/day). These results suggest that within the release rate range of 5–30 mg/day, *M. aeneus* may respond nonspecifically to all, or at least a large number of, isothiocyanates. In this, the beetle differs from other brassica-associated insects. Two species of *Phyllotreta* flea beetles, the northern false chinch bug, *Nysius niger*, the brassica pod midge, *Dasineura brassicae*, and two parasitoids, *Meteorus leviventris* and *Platygaster subuliformis*, show specificity in response to different isothiocyanate analogs released at similar rates (Pivnick, 1993; Pivnick et al., 1991, 1992; Murchie et al., 1997). *Phyllotreta cruciferae* and *P. striolata* are attracted preferentially to allyl NCS, to a lesser degree to benzyl and ethyl NCS, and not at all to five other isothiocyanates, when released at 4 mg/day (Pivnick et al., 1992).

High release rates of isothiocyanates remain attractive to *M. aeneus*, in contrast to other insect species where isothiocyanates become repellent (or inactive) when an upper limit is reached (Pivnick, 1993). *M. leviventris* is attracted to allyl NCS released at 4 mg/day but not at 40 mg/day. *M. aeneus* also responds to the release of submilligram amounts of isothiocyanates. We found that a mixture of 3-butenyl, 4-pentenyl, and 2-phenylethyl NCS, total release rate 400–500 μg/day, was as attractive as the mixture released at 15 mg/day.

The present results confirm that both visual and odor cues affect the orientation of *M. aeneus* to traps. Attraction to the most effective unbaited yellow traps is enhanced up to 3.3 times by the addition of an isothiocyanate lure. However the significant visual $\times$ odor interactions observed in two experiments (2, 4) indicate that the magnitude of the odor effect is dependent on the nature of the visual cue. Lures had the greatest effect on traps of unattractive orientation or color. Nevertheless, baited traps that were visually unattractive still caught few insects. This suggests that visual cues may be more important than odor cues in the orientation of *M. aeneus*, although their relative importance may vary in a complex manner with the distance from the food or oviposition resource, as has been observed with the onion fly, *Delia antiqua* (Judd and Borden, 1991) and the apple maggot fly, *Rhagoletis pomonella* (Green et al., 1994).

There are similarities and differences in the response of *M. aeneus* and *C. assimilis* to visual and odor cues. Both species respond similarly to the different trap types and both are attracted predominantly to yellow (Smart et al., 1997; this report), but no statistically significant interactions are observed between the visual and olfactory cues that attract the seed weevil (Smart et al., 1997). There are also differences between the responses of the two species to isothiocyanates. Whereas nondiapausing *M. aeneus* were always attracted to all the isothiocyanates tested, *C. assimilis* was largely unresponsive to allyl NCS (Smart et al., 1993, 1995, 1997; this report). It was attracted only during the spring

and autumn migratory phases to 2-phenylethyl NCS and to the mixture of allyl, 3-butenyl, 4-pentenyl, and 2-phenylethyl NCS (Smart et al., 1997; Smart and Blight, 1997).

The work reported here has led to successful development of a trap for monitoring *M. aeneus* and *C. assimilis* in the spring (Smart et al., 1996). The information is also essential for the development of control strategies, such as the push–pull technique (Smart et al., 1994), and for the design of insect-resistant genetically modified oilseed crops. Although our results suggest that neither modification of crop color nor of glucosinolate profile is likely to have an effect on the orientation of *M. aeneus*, other changes to the odor profile of the crop might, because the antenna of the beetle perceives at least 23 additional oilseed rape volatiles (Blight et al., 1995). Accordingly, the effect of a variety of volatile host and nonhost compounds on the behavior of the beetle was studied in concurrent field experiments. These results will be reported in another paper.

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INFLUENCE OF PRETREATMENT STRESSES ON INHIBITORY EFFECTS OF FERULIC ACID, AN ALLELOPATHIC PHENOLIC ACID

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Abstract—Experiments were conducted to determine the potential for acclimation (i.e., increased tolerance) to allelopathic phenolic acids resulting from pretreatment of seedlings with allelochemical (ferulic acid), drought, or nutrient stress. Cucumber seedlings were exposed to pretreatment stresses in a nutrient culture system for nine days, starting with day 3. Seedlings were subsequently treated for 5 hr with 0, 0.25, 0.5, or 0.75 mM ferulic acid. Acclimation (tolerance) was quantified by percentage inhibition of net phosphorus uptake. Seedlings grown with ferulic acid or drought pretreatment stresses were more tolerant to subsequent ferulic acid treatments (i.e., inhibition of net phosphorus uptake by ferulic acid was reduced). Nutrient pretreatment stress eliminated the inhibitory activity of ferulic acid on net phosphorus uptake. The results suggest that a general acclimation response to a variety of pretreatment stresses can confer an increased tolerance of plants to allelopathic phenolic acids such as ferulic acid.

Key Words—Allelopathy, ferulic acid, pretreatment, acclimation, tolerance, drought stress, nutrient stress, *Cucumbis sativus*, cucumber.

INTRODUCTION

In nature, plants are continuously exposed to allelopathic compounds, as well as to other environmental stresses (e.g., drought, nutrient stress). Most research

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examining the interaction of allelopathy and environmental stress focuses on how allelochemical production in source plants is altered by stress (for reviews, see Gershenzon, 1984; Tang et al., 1995). A limited number of studies, however, have addressed the question of how the tolerance of recipient plants to allelochemicals is influenced by other environmental stress factors. Plant tolerance to allelochemicals has been shown to be modified by high and low temperature stress (Glass, 1976; Einhellig and Eckrich, 1984), drought stress (Duke et al., 1983; Einhellig, 1987), and nutrient stress (Glass, 1976; Stowe and Osborn, 1980; Hall et al., 1982, 1983; Vaughan and Ord, 1991). All of these studies, however, involved exposures of plants grown under "ideal experimental conditions" to allelochemicals and other concurrent stresses (temperature, drought, nutrient stress), or in one instance, to a two-day nutrient stress prior to the introduction of allelochemicals (Stowe and Osborn, 1980). Thus, the role of stressful pretreatment environments on plant tolerances to allelochemicals has not been adequately characterized.

Acclimation to one environmental stress may result in acclimation to others. For example, salt treatment can increase tolerance to chilling (Riken et al., 1976) and frost (Schmidt et al., 1986). This "cross-tolerance" acclimation may be due to a centralized plant stress response system (Chapin, 1991; Luo et al., 1993).

This study was conducted to determine the potential for a general acclimation response to a variety of pretreatment stresses (allelochemicals, drought, nutrient stress) that would confer an increased tolerance of plants to allelopathic phenolic acids. Cucumber (*Cucumis sativus* cv. Early Green Cluster) was chosen as the bioassay species because the inhibitory effects of phenolic acids on water utilization, net nutrient uptake, and growth have been characterized in considerable detail (Blum and Dalton, 1985; Blum and Rebbeck, 1989; Booker et al., 1992). Nutrient culture was used for precise control of solution concentrations surrounding roots. Ferulic acid was used as a representative allelochemical that is commonly found in plants (Siqueira et al., 1991; Harborne, 1993), leaf leachates (Abdul-Rahman and Habig, 1989), root exudates (Tang and Young, 1982; Tang, 1986), plant debris (Kuiters, 1990; Blum et al., 1991), and soils (Whitehead et al., 1982, 1983; Kuiters and Denneman, 1987; Blum et al., 1991).

METHODS AND MATERIALS

General Aspects. Cucumber seeds (*Cucumis sativus* cv. Early Green Cluster) were germinated in the dark in trays of vermiculite at 30°C for two days. The seedlings were exposed to 11 hr of light and 12 hr of dark before transplanting into the designated growth media or hydroponic solutions described for the specific experiments. Prior to treatment, seedlings were grown under the specified pretreatment condition for nine days in a growth chamber in the Southeastern Plant Environmental Laboratory at a PPFD of 400 μmol/m^2/sec (supplied by fluorescent and

incandescent lights), a 12-hr photoperiod, and a 26/22°C day–night temperature (NCSU Phytotron) (Downs and Thomas, 1991). Deionized water was added daily to replace water lost by evapotranspiration. For plants grown in nutrient solutions, the solutions were completely changed every two days after transplanting. Nutrient solutions were replaced with 0.5 mM $CaSO_4$ solutions one day before experimental treatments began. Seedlings were treated in a system of small plastic beakers (50 ml) enclosed within a larger square container. Roots were suspended through a hole in the lid of the large container and placed into 40 ml of solution(s) (pH 5.5) in the 50-ml beakers. The large container was wrapped in foil to exclude light from the system. In addition to the stated concentration(s) of ferulic acid, all treatment solutions contained 0.5 mM $CaSO_4$, 5 mM 2(*N*-morpholino)ethanesulfonic acid (MES buffer), and 0.5 mM KH_2PO_4. Preliminary experiments indicated that 5 mM MES buffer had no detectable effect on net phosphorus or water uptake and that the buffer adequately stabilized the pH at 5.5 during the time frame of an experiment (5 hr). The net uptake of phosphorus (Pi) and water were based on depletion of these substances from solution. The Pi remaining at the end of the experiments was determined by a spectrophotometric method (Taussky and Shorr, 1953). The water remaining was measured to the nearest 0.5 ml in a graduated cylinder and was used in calculations of the amount of Pi remaining in solution at the end of the 5-hr treatment. Preliminary experiments indicated that an aerated system utilizing 40 ml of solution per cup and a 5-hr treatment period provided adequate Pi uptake for detection, without limiting Pi availability by the end of the time period. All uptake values were expressed on a per dry root weight basis since preliminary experiments indicated that root dry weight was closely related to root fresh weight [mg root dry wt = −0.06 + 0.02 (mg root fresh wt); $P < 0.0001$; $R^2 = 0.94$], root surface area [mg root dry wt = 3.13 + 1.55 (root surface area in cm^2); $P < 0.0001$; $R^2 = 0.90$], and root volume [mg root dry wt = −1.35 + 20.00 (root volume in ml); $P < 0.0001$; $R^2 = 0.87$]. Fresh weights of dry-blotted roots were determined gravimetrically immediately after harvest. Dry weights were determined gravimetrically after oven-drying at 40°C for 48 hr. Root surface area was estimated by direct readings from a leaf area meter. Root volume was measured by the displacement of water in a closed volumeter tube system modeled after Novoselov (1960).

Ferulic Acid Pretreatment. Seedlings were pretreated for nine days (starting with day 3) in 110 ml of Hoagland's solution (pH 5.5) (Hoagland and Arnon, 1950) containing 0, 0.1, 0.2, or 0.4 mM of ferulic acid (PRE-FER). Following pretreatment and a one-day exposure to 0.5 mM $CaSO_4$, seedlings were treated for 5 hr in solutions (40 ml) containing 0, 0.25, 0.5, or 0.75 mM ferulic acid (FER). Treatments were replicated four times ($N = 64$). In a second ferulic acid experiment, seedlings that had been pretreated in 0 or 0.4 mM PRE-FER were subsequently treated in a split-root system, where portions of the root system (approximately none, 1/4, 1/2, 3/4, all) were exposed to treatment solutions

containing 0.5 mM FER. The remainder of the root system was placed in a second container with 0 mM FER (Lehman et al., 1994).

Drought Stress Pretreatment. Seedlings were pretreated for nine days (starting with day 3) in 110 ml of Hoagland's solution containing 0%, 0.25%, 0.5%, 1%, or 2% polyethylene glycol (PEG), molecular weight = 8000. The freezing point depressions (determined on an Osmette Precision Osmometer; Precision System Inc., Natick, Massachusetts) for the pretreatment solutions were 27.2, 29.0, 31.4, 34.2, and 37.6 mOsm, for the 0, 0.25, 0.5, 1, and 2% PEG, respectively. Following pretreatment and a one-day exposure to 0.5 mM $CaSO_4$, seedlings were treated for 5 hr in solutions (40 ml) containing 0, 0.25, 0.5, or 0.75 mM FER. There were three replicates in this experiment, for a total of 64 plants.

Nutrient Stress Pretreatment. During the nine-day pretreatment period, seedlings were grown in 110 ml of various dilutions of Hoagland's solution (full-, 1/4-, 1/16-, 1/32-, or 1/64-strength). Following pretreatment and a one-day exposure to 0.5 mM $CaSO_4$, seedlings were treated for 5 hr in solutions (40 ml) containing 0, 0.25, 0.5, or 0.75 mM FER and 0.5 mM KH_2PO_4. Treatments were replicated three times ($N = 64$). In a second experiment, seedlings were grown in 110 ml of Hoagland's solution that varied only in the amounts of KNO_3 and $Ca(NO_3)_2$ (full-, 1/4-, 1/16-, 1/32-, or 1/64-strength); reductions in the amounts of KNO_3 and $Ca(NO_3)_2$ were substituted by K_2SO_4 and $CaSO_4$ to keep the supply of K and Ca constant across all groups. This experiment used the same treatment protocol as the first.

Growth Media Pretreatment. During the nine-day pretreatment period, seedlings were grown either in jars containing 110 ml of Hoagland's solution, or in Styrofoam cups (225 ml) containing sand or gravel. Prior to planting the seedlings, the sand and gravel were washed in deionized water and sieved through an 840-μm (sand) or 5-mm (gravel) mesh screen. Seedlings were subsequently treated in a split-root system, where portions of the root system (approximately none, 1/3, 2/3, all) were exposed to treatment solutions containing 0.5 mM FER. The remainder of the root system was placed in a second container with 0 mM FER (Lehman et al., 1994). A total of 48 plants was used.

Data Analysis. All experiments used a completely randomized block design. Because the effect of blocking was insignificant for all experiments, blocks were not included as a factor in the final analyses. Control (0 mM FER treatment) means for Pi uptake/g root dry weight were determined for each pretreatment group (e.g., nutrient solution strength, growth medium) and used to calculate the percentage of inhibition of Pi uptake for seedlings treated in 0.25–0.75 mM FER as follows:

$$\frac{\text{control mean Pi uptake/g root} - \text{Pi uptake/g root of treatment}}{\text{control mean Pi uptake/g root}} \times 100$$

Data were subjected to analysis of variance and subsequent regression analyses using the JMP statistical package (SAS Institute, Inc., 1994). Statistical significance was based on an alpha of 0.05 or less. For split-root experiments, the proportion of roots exposed to FER was based on the actual value derived from dry weights of the roots.

RESULTS

Ferulic Acid Pretreatment. In the first experiment (whole-root treatments), pretreatment of cucumber seedlings for nine days with 0.1, 0.2, or 0.4 mM ferulic acid (PRE-FER) did not alter the net phosphorus (Pi) uptake per gram root dry weight of these seedlings when compared to the pretreatment controls (0 mM PRE-FER). Subsequent treatment of these control and pretreated seedlings with increasing concentrations of ferulic acid (FER) (0.25, 0.5, or 0.75 mM FER) linearly reduced net Pi uptake per gram root dry weight of seedlings from all pretreatments groups (0, 0.1, 0.2, and 0.4 mM PRE-FER), compared to treatment control (0 mM FER) seedlings (Table 1). The phytotoxicity of FER was characterized by calculating the percentage inhibition of net Pi uptake for each PRE-FER group by using its 0 mM FER treatment as control. Percent inhibition of net Pi uptake varied among pretreatment groups (significant ANOVA interaction of treatment and pretreatment effects; $P = 0.0208$). The regression models for percentage inhibition of net Pi uptake by FER concentration were significant only for the 0 mM PRE-FER group (Table 2), for which percentage inhibition of net Pi uptake increased linearly from 29.8 ± 4.5 (mean ± standard error) for 0.25 mM FER to 59.7 ± 4.6 for 0.75 mM FER. Increasing concentrations of FER, however, did not modify the percentage inhibition of net Pi uptake for the 0.1, 0.2, and 0.4 mM PRE-FER pretreatment groups; maximum inhibition was already obtained with the lowest treatment concentration used, 0.25 mM FER. The mean percentage inhibitions of net Pi uptake were 41.3 ± 5.3, 33.8 ± 5.5, and 22.7 ± 4.7 for 0.1, 0.2, and 0.4 mM PRE-FER, respectively. In the second experiment (split-root treatments), pretreatment of seedlings for nine days with 0.4 mM PRE-FER did not alter the net Pi uptake per gram root dry weight of these seedlings when compared to the pretreatment controls (0 mM PRE-FER). When subsequently treated with 0.5 mM FER, the regression models for net Pi uptake per gram root dry weight by proportion of roots treated was significant for the 0 mM PRE-FER pretreatment group (Table 1); there was no significant effect on net Pi uptake by 0.5 mM FER treatment for the 0.4 mM PRE-FER group. Likewise, the regression model for percentage inhibition of net Pi uptake by proportion of roots treated was significant only for the 0 mM FER pretreatment group (Table 2), with the percentage inhibition of net Pi uptake increasing linearly as the proportion of root treated with FER was increased. The maximum

TABLE 1. PARTIAL REGRESSION COEFFICIENTS, R^2, AND P VALUES FOR PHOSPHORUS UPTAKE[a] BY CUCUMBER SEEDLINGS

Experiment	Pretreatment[b]	Intercept	Linear[c]	P	R^2
Ferulic acid					
Whole-root[d]	0 mM PRE-FER	3.87	−3.27 A	0.0000	0.76
	0.1 mM PRE-FER	3.86	−2.47 AB	0.0072	0.41
	0.2 mM PRE-FER	3.96	−2.19 B	0.0053	0.44
	0.4 mM PRE-FER	3.64	−1.50 B	0.0026	0.54
Split-root[e]	0 mM PRE-FER	3.49	−1.90	0.0000	0.73
	0.4 mM PRE-FER			NS	
Drought stress[d]	0% PEG	3.90	−2.92 A	0.0000	0.87
	0.25% PEG	4.43	−3.11 B	0.0000	0.82
	0.5% PEG	3.83	−1.41 C	0.0322	0.38
	1.0% PEG	3.99	−1.63 C	0.0010	0.68
Nutrient stress[d]	1 × Hoagland's	4.39	−3.69 A	0.0001	0.80
All nutrients	1/4 × Hoagland's	4.30	−3.17 A	0.0000	0.83
	1/16 × Hoagland's			NS	
	1/32 × Hoagland's			NS	
	1/64 × Hoagland's			NS	
Nitrate reduced	1 × nitrate	3.71	−2.03 A	0.0029	0.61
	1/4 × nitrate	3.87	−3.64 B	0.0003	0.74
	1/16 × nitrate			NS	
	1/32 × nitrate			NS	
	1/64 × nitrate			NS	

[a]Phosphorus uptake measured in milligrams per gram dry root weight.
[b]PRE-FER = ferulic acid pretreatment; PEG = polyethylene glycol.
[c]Slopes within an experiment not followed by the same letter are significantly different.
[d]Treatment with 0, 0.25, 0.5, and 0.75 mM ferulic acid (FER); dependent variable = FER concentration.
[e]Treatment with 0.5 mM FER; dependent variable = proportion roots treated.

mean percentage inhibition of net Pi uptake was 55.0 ± 3.4, which was obtained for the 0 mM PRE-FER group when 100% of the root system was treated in FER.

Drought Stress Pretreatment. Pretreatment of seedlings with 2% polyethylene glycol (PEG) was lethal or caused extensive shoot damage. Thus, these plants were excluded from subsequent treatments. Pretreatment of seedlings with 0.25, 0.5, or 1.0% PEG did not alter the net Pi uptake per gram root dry weight of these seedlings, compared to the pretreatment controls (0% PEG). Subsequent treatment of these control and pretreated seedlings with increasing concentrations of FER (0.25, 0.5, or 0.75 mM FER) linearly reduced net Pi uptake per gram root dry weight of seedlings from all pretreatment groups (0, 0.25, 0.5, and 1.0% PEG), compared to treatment control (0 mM FER) seedlings (Table 1). The phy-

TABLE 2. PARTIAL REGRESSION COEFFICIENTS, R^2, AND P VALUES FOR PERCENTAGE INHIBITION OF NET PHOSPORUS UPTAKE BY CUCUMBER SEEDLINGS

Experiment	Pretreatment[a]	Intercept	Linear[b]	P	R^2
Ferulic acid					
Whole-root[c]	0 mM PRE-FER	14.01	63.79	0.0014	0.66
	0.1 mM PRE-FER			NS	
	0.2 mM PRE-FER			NS	
	0.4 mM PRE-FER			NS	
Split-root[d]	0 mM PRE-FER	21.19	32.47	0.0063	0.45
	0.4 mM PRE-FER			NS	
Drought stress[c]	0% PEG	2.64	71.16 A	0.0016	0.83
	0.25% PEG	5.58	62.17 A	0.0142	0.60
	0.5% PEG			NS	
	1.0% PEG			NS	
Nutrient stress[c]	1 × Hoagland's	27.90	43.16 A	0.0061	0.68
All nutrients	1/4 × Hoagland's	−10.13	88.35 B	0.0029	0.74
	1/16 × Hoagland's			NS	
	1/32 × Hoagland's			NS	
	1/64 × Hoagland's			NS	
Nitrate reduced	1 × Nitrate	5.75	46.97 A	0.0110	0.63
	1/4 × Nitrate	3.92	88.39 B	0.0254	0.53
	1/16 × Nitrate			NS	
	1/32 × Nitrate			NS	
	1/64 × Nitrate			NS	

[a]PRE-FER = ferulic acid pretreatment; PEG = polyethylene glycol.
[b]Slopes within an experiment not followed by the same letter are significantly different.
[c]Treatment with 0, 0.25, 0.5, and 0.75 mM ferulic acid (FER); dependent variable = FER concentration.
[d]Treatment with 0.5 mM FER; dependent variable = proportion roots treated.

totoxicity of FER was characterized by calculating the percentage inhibition of net Pi uptake for each PEG pretreatment group by using the appropriate group 0 mM FER treatment as control. Percent inhibition of net Pi uptake varied among pretreatment groups (significant ANOVA interaction of treatment and pretreatment effects; $P = 0.0184$). The regression models for percentage inhibition of net Pi uptake by FER concentration were only significant for the 0 and 0.25% PEG pretreatments (Table 2), with the percentage inhibition of net Pi uptake increasing linearly as the treatment FER concentration was increased; the mean percentage inhibition ranged from 24.0 ± 5.7 (0.25 mM FER treatment) to 57.9 ± 2.7 (0.75 mM FER treatment) for the 0% PEG pretreatment group and ranged from 23.6 ± 3.8 (0.25 mM FER treatment) to 53.2 ± 1.8 (0.75 mM FER treatment) for the 0.25% PEG pretreatment group. Increasing concentrations of FER,

however, did not modify the percentage inhibition of net Pi uptake for the 0.5 and 1.0% PEG pretreatment groups. Maximum inhibition was already obtained with the lowest treatment concentration used (0.25 mM FER). The mean percentage inhibition of net Pi uptake was 28.3 ± 2.7 and 23.8 ± 3.1 for the 0.5% and 1.0% PEG pretreatments, respectively.

Nutrient Stress Pretreatment. Pretreatment of seedlings with 1/4-, 1/16-, 1/32-, or 1/64-strength Hoagland's solution (HOAG) or reduced-nitrate Hoagland's solution (N-HOAG) did not alter the net Pi uptake per gram root dry weight of these seedlings, compared to the pretreatment controls (full-strength HOAG or N-HOAG). Responses of these control and pretreated seedlings to increasing concentrations of FER (0.25, 0.5, or 0.75 mM FER) varied among pretreatment groups (significant ANOVA interactions of treatment and pretreatment effects with $P = 0.0289$ and 0.0002 for first and second experiments, respectively). Increasing concentrations of FER linearly reduced net Pi uptake per gram root dry weight of seedlings for the full-strength and 1/4-strength HOAG or N-HOAG pretreatments (Table 1). Increasing concentrations of FER, however, did not modify the net Pi uptake per gram root dry weight for the 1/16-, 1/32-, and 1/64-strength HOAG or N-HOAG pretreatments. For seedlings pretreated with full-strength and 1/4-strength solutions, the percentage inhibition of net Pi uptake increased linearly as the FER concentration was increased (Table 2). In the first experiment (HOAG, nutrient concentration dilutions), the mean percentage inhibition ranged from 40.2 ± 5.0 (0.25 mM FER) to 60.7 ± 2.6 (0.75 mM FER) for the full-strength Hoagland's pretreatment group and ranged from 18.2 ± 5.5 (0.25 mM FER) to 60.6 ± 5.4 (0.75 mM FER) for the 1/4-strength Hoagland's pretreatment group. In the second experiment (N-HOAG, nitrate concentration dilutions), the mean percentage inhibition ranged from 15.5 ± 6.9 (0.25 mM FER) to 39.0 ± 4.0 (0.75 mM FER) for the full-strength nitrate pretreatment group and ranged from 14.3 ± 3.2 (0.25 mM FER) to 58.5 ± 1.7 (0.75 mM FER) for the 1/4-strength nitrate pretreatment group. In the first experiment, mean net Pi uptake (in milligrams) per gram dry root weight for seedlings grown in 1/16-, 1/32-, and 1/64-strength HOAG were 3.3 ± 0.3, 4.0 ± 0.4, and 3.3 ± 0.2, respectively; these were not significantly different from 3.9 ± 0.2, the mean net Pi uptake of controls (0 mM FER treatment). In the second experiment, mean net Pi uptake (in milligrams) per gram dry root weight for seedlings grown in 1/16-, 1/32-, and 1/64-strength N-HOAG were 3.6 ± 0.3, 3.0 ± 0.2, and 3.3 ± 0.2, respectively; these were not significantly different from 3.5 ± 0.2, the mean net Pi uptake of controls (0 mM FER treatment).

Growth Media Pretreatment. There was no significant difference in net Pi uptake per gram root dry weight or percentage inhibition of net Pi uptake by 0.5 mM FER for seedlings grown in sand, gravel, or nutrient culture (i.e., ANOVA showed no significant pretreatment main effects or treatment–pretreatment interactions). Thus, the linear relationship between the proportion of roots treated in 0.5 mM FER and net Pi uptake of plant per gram root dry weight was described

by a single equation: net Pi uptake (in milligrams) per gram root = 4.19 − 2.08 (proportion roots treated); $P = 0.0000$; $R^2 = 0.49$. Likewise, the percentage inhibition net Pi uptake of seedlings was described as follows: percentage inhibition net Pi uptake = −6.82 + 54.55 (proportion roots treated); $P = 0.0000$; $R^2 = 0.67$.

DISCUSSION

The phytotoxicity of ferulic acid (FER) treatments was characterized by using the percentage inhibition of net phosphorus (Pi) uptake as the criterion. Percent inhibition of net Pi uptake for seedlings grown under ferulic acid (0.1, 0.2, or 0.4 mM PRE-FER), drought (0.5% or 1.0% PEG), or nutrient (1/16-, 1/32-, 1/64-strength HOAG or N-HOAG) pretreatment stresses were reduced or eliminated for increasing FER treatments, indicating that acclimation (i.e., increased FER tolerance) had occurred. For ferulic acid and drought pretreatment stresses, maximum inhibition occurred at the lowest concentration of FER, but this inhibition was less than the maximum inhibition of nonacclimated control seedlings (0 mM PRE-FER or 0% PEG pretreatments). For nutrient pretreatment stress of ≤1/16-strength HOAG or N-HOAG, subsequent FER treatment effects were eliminated [i.e., net Pi uptake per gram root dry weight of nutrient stress pretreated seedlings was not affected by FER treatment, but net Pi uptake per gram root dry weight of control seedlings (i.e., no pretreatment) was inhibited by FER treatment]. The 1/4 strength HOAG pretreatment appeared to be a transition stage for acclimation since the percentage inhibition net Pi uptake was reduced from that of controls (full-strength HOAG) at the lowest FER treatment concentration (0.25 mM), but not at higher FER concentrations. This "transition stage" for acclimation was not observed for the 1/4-strength N-HOAG pretreatment. In fact, seedlings pretreated in 1/4-strength N-HOAG showed a higher maximum percentage inhibition of net Pi uptake than the controls (full-strength N-HOAG). The biological significance of this observation may be questioned due to the low maximum inhibition observed for controls in this experiment (38.99 ± 3.97%) compared to those in all other experiments (ranged from 55.04 ± 3.39% to 60.68 ± 2.60%). The data suggest that pretreatment stresses must exceed some threshold for some pretreatment stresses (e.g., >0.25% PEG; >1/4 strength HOAG or N-HOAG) but not necessarily for others (e.g., for PRE-FER) before acclimation of seedlings to increasing concentrations of FER can occur.

We suspect that the two previous attempts (Blum and Dalton, 1985; Lehman et al., 1994) to demonstrate acclimation to allelopathic phenolic acids by using ferulic acid pretreatments were unsuccessful because the pretreatment periods used in those studies were too short (<4 days) (Lehman et al., 1994) and a less

sensitive indicator (inhibition of leaf expansion) was monitored. The need for a sensitive response variable in the detection of acclimation due to PRE-FER is consistent with observations by Holappa and Blum (1991). These researchers presented evidence of acclimation by observing that endogenous abscisic acid (ABA) concentrations were affected only by the first ferulic acid treatment. ABA levels did not appear to be affected by subsequent FER treatments. The experiments by Holappa and Blum (1991) and those reported here indicate that cucumber seedlings do acclimate to phenolic acids and, that once acclimated, they become more tolerant to subsequent phenolic acid exposure.

The results of this study also suggest that acclimation to phenolic acids may be a general stress response since acclimation, based on percentage inhibition of net Pi uptake, was achieved by a variety of pretreatment stresses (e.g., nutrient or drought) other than ferulic acid (PRE-FER) itself. This centralized stress response, hypothesized by Chapin (1991), may be mediated primarily by ABA, since pretreatment stresses have been found to be coupled to increasing ABA levels in plants (Chapin et al., 1988; Holappa and Blum, 1991; Luo et al., 1993). ABA is known to enhance tolerance to a variety of stresses (Daie and Campbell, 1981; Zeevaart and Creelman, 1988; Luo et al., 1993). This may be due, in part, to the influence of ABA on the expression of a number of genes (Cohen and Bray, 1990; Skriver and Mundy, 1990; Kahn et al., 1993; Leone et al., 1994), resulting in the preferential synthesis of "stress proteins" (Ho and Sachs, 1989; Luo et al., 1993; Leone et al., 1994). It is also possible that a number of other physiological, morphological, and/or anatomical changes are involved in acclimation. These changes may also be mediated by ABA and/or by other plant growth regulators. For example, ethylene production is also known to increase under a variety of stresses (Abeles, 1973; Morgan and Drew, 1997). It is likely that several plant growth regulators are involved since some studies indicate that ABA alone cannot account for a centralized stress response (Leone et al., 1994; Coleman and Schneider, 1996).

In summary, this study demonstrates the potential ability in cucumber seedlings for a variety of physical and chemical pretreatment stresses (allelochemical, drought, nutrient stress) to induce acclimation (i.e., increased tolerance) to phenolic acids, if the pretreatment stresses are adequate (i.e., long and severe enough). For example, the severity of pretreatment stress necessary to induce acclimation of cucumber seedlings to phenolic acids varied in these experiments in the following ways. Adequate pretreatments for acclimation were between 0 and 0.1 mM PRE-FER for phenolic acid stress, between 0.25% and 0.5% PEG (29.0–31.4 mOsm) for drought stress, and between 1/4- and 1/16-strength HOAG or N-HOAG for nutrient stress. This may help to explain why plants grown under field conditions are more tolerant (or less sensitive) to phenolic acid compounds compared to plants grown in growth chambers or greenhouses.

Two additional factors (substrate and root–phenolic acid contact) that might influence the acclimation process of cucumber seedlings to phenolic acids were also included in this study. The "stress" induced by growing cucumber roots in sand or gravel (pretreatment) compared to nutrient solution did not modify the behavior of cucumber seedlings to subsequent FER treatment. This suggests that the acclimation of cucumber seedlings to phenolic acids that was observed in nutrient culture should be directly relevant to seedlings grown in solid substrates (i.e., no difference would be expected when seedlings are grown in solution or substrate). Finally, the similarity in behavior of both the whole-root and the split-root system suggests that acclimation, just like phenolic acid effects, is a reversible (Glass, 1973, 1974; Blum and Dalton, 1985), localized phenomenon at the root level (Lyu and Blum, 1990; Lehman et al., 1994). The present study, however, does not address how readily acclimation to phenolic acids may be reversed.

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DISCRIMINATIVE RESPONSE TO ANIMAL, BUT NOT PLANT, CHEMICALS BY AN INSECTIVOROUS, ACTIVELY FORAGING LIZARD, *Scincella lateralis*, AND DIFFERENTIAL RESPONSE TO SURFACE AND INTERNAL PREY CUES

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Abstract—Responses by the insectivorous, actively foraging scincid lizard, *Scincella lateralis*, to chemical cues from a plant food favored by herbivorous lizards, its ability to discriminate prey chemicals from control substances, and its relative response to internal and surface prey chemicals were studied experimentally. We presented chemical cues to the lizards on cotton swabs and recorded their tongue-flicks and biting attacks on the swabs. The lizards exhibited significantly greater tongue-flick rates and biting frequencies to prey surface cues than to plant surface chemicals from romaine lettuce, diluted cologne (pungency control), and deionized water. Responses to the plant stimuli did not differ from those to the two control stimuli, in contrast with strong responses to the same plant cues by herbivores. This finding provides the first information suggesting that chemosensory response may be adapted to diet, with responsiveness to plant stimuli evolving de novo in herbivores. Biting and tongue-flicking responses were significantly greater to cricket chemicals than to all other stimuli, among which there were no differences. Thus, the lizards are capable of prey chemical discrimination, which may be ubiquitous among actively foraging lizards. The lizards exhibited more frequent biting and higher tongue-flick rates to internal than surface prey chemicals. Although different methods of stimulus preparation are appropriate for different purposes, we conclude that prey surface chemicals available to foraging lizards are most desirable for studies bearing on location and identification of prey.

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Key Words—Behavior, chemical senses, tongue-flicking, diet, Squamata, Scincidae, *Scincella lateralis*.

INTRODUCTION

Use of senses to locate, identify, and evaluate food and its relationship to hunting methods in squamate reptiles are among the best known aspects of vertebrate chemical ecology. In snakes there is considerable evidence that sensory responsiveness to prey chemicals is adjusted to variation in diet. Chemosensory responses to prey chemicals by garter snakes (*Thamnophis*) are strongest to preferred prey and vary concordantly with geographic variation in diet within and between species (Burghardt, 1969; Arnold, 1981a,b). In *T. elegans*, differences among populations in feeding and chemosensory responses to prey are heritable in a manner suggesting that the responses are adaptively adjusted to match diet in local populations (Arnold, 1981a,b).

Like snakes, a wide variety of actively foraging lizards can discriminate prey chemicals from control substances by tongue-flicking (Cooper, 1994a,b, 1995, 1997), which strongly suggests that the discriminations involve vomerolfaction (Cooper and Burghardt, 1990a; Halpern 1992). Correlated evolution has occurred between prey chemical discrimination and foraging mode (Cooper, 1995) and between prey chemical discrimination and both lingual structure and abundance of vomeronasal chemoreceptor cells (Cooper, 1997).

Whether lizard chemosensory discriminations are adjusted to diet is unknown and cannot be readily determined for animal prey because most lizards are generalist predators. However, several species of herbivores and omnivores respond strongly to plant chemicals, but retain responsiveness plesiomorphically to animal prey cues (Cooper and Alberts, 1990; Cooper, unpublished data). If responsiveness to plant chemicals has evolved de novo in herbivores as an adjustment to diet, it must be absent in insectivores. Many lizards eat only animal prey, but some insectivores occasionally eat plant matter (e.g., Vitt and Cooper, 1986; Dearing, 1993). We experimentally assessed the chemosensory response to food by an actively foraging, insectivorous species, *Scincella lateralis*, belonging to a taxon known for widespread occasional plant consumption (Vitt and Cooper, 1986). Because these lizards rarely consume plants, we predicted lack of response to chemical stimuli from a plant readily consumed by herbivorous lizards.

An additional poorly known aspect of squamate responses to food chemicals is the relationship between the method of stimulus preparation and response strength. We used the same lizards to study effects of use of internal versus surface prey cues on chemosensory responses. In a widely used method, chemical stimuli are presented to squamates on cotton swabs to gauge chemosensory

responses to food (e.g., Burghardt, 1970a,b; Cooper, 1994a,b, 1998a; Cooper and Burghardt, 1990b). Despite wide use of this method, the variation in effectiveness of stimuli with method of stimulus preparation has received scant attention. Burghardt (1969, 1970a, 1973) prepared aqueous extracts of prey surface chemicals by heating whole prey in a standardized manner. By diluting the standard extract with water, he showed that response strength varied directly with extract concentration (Burghardt and Hess, 1968).

Because prey surface chemicals may differ in concentration and content from internal chemicals, differences in preparation methods might yield quantitative and qualitative differences in results. For example, geckos of the genus *Coleonyx* often bite without first tongue-flicking when responding to aqueous extracts of macerated crickets or body fluids of crushed mealworms (Dial, 1978; Dial et al., 1989; Dial and Schwenk, 1996). In contrast, tongue-flicking precedes biting in a large majority of the geckos *Eublepharis macularius* and *C. variegatus* when stimuli are prepared by rolling a wet swab over the body surface of prey (Cooper, 1998b). We tested chemosensory responses of *S. lateralis* to surface and internal prey chemicals as a likely source of response differences.

METHODS AND MATERIALS

Scincella lateralis is a lygosomine scincid lizard from the eastern United States. It is an active forager (Brooks, 1967; our unpublished observations) that searches for prey in ground surface litter (Lewis, 1951; Brooks, 1967), where it consumes termites and other hidden prey (Lewis, 1951; Hamilton and Pollack, 1961; Brooks, 1963). Adult *S. lateralis* were collected in Dallas, Texas, in September 1998 and transported to Indiana University Purdue–University Fort Wayne (IPFW). They were kept in an accredited animal care facility where each lizard was housed alone in a translucent plastic terrarium (41 × 28 × 23 cm) having a rock, three moist paper towels, a water bowl, and a plastic top. The ambient temperature was 29°C and a 12L : 12D cycle was maintained by fluorescent bulbs. Testing began on the second day at IPFW. Lizards were fed crickets upon completion of the experiment on food chemical discrimination, which required two to four days, and received no food for two days prior to the other experiment.

We conducted experiments to assess the ability of the skinks to discriminate animal prey from several control simuli by using only chemical cues and to investigate possible differences between responses to internal and body surface chemical cues from prey. Stimuli were presented on cotton applicators. For all stimuli, the first step of stimulus preparation was to dip the cotton swab into deionized water, which served as an odorless control. After the swab was moistened, we added another stimulus to the cotton (romaine lettuce, cricket, cologne),

if needed. A pungency control for response to an odorous, but trophically irrelevant, stimulus was prepared by dipping the swab into diluted cologne (Mennen Skin Bracer, Spice Scent, 3 : 1 deionized water to cologne). The swab was rolled across the surface of a leaf of romaine lettuce to test possible response by the insectivorous skinks to a plant food consumed avidly by many herbivorous lizards. Prey stimuli from domestic cricket (*Acheta domesticus*) were prepared by rolling the swab over the integument (external stimuli) or over tissues extruded from a freshly squashed cricket. Visible internal materials from crickets adhering to swabs were removed prior to testing.

Cologne has been used often as a pungency control, but can be aversive in high concentrations (Dial and Schwenk, 1996; Cooper, 1998a,b). Aversion to cologne can be eliminated by dilution (Cooper, 1998a,b). For insectivores, chemicals from nonnoxious plants provide an independent pungency control that can shed light on responses to both prey cues and possible negative responses to cologne. Detection can be inferred from significantly different responses to food stimuli and the odorless control, but the possibility remains that the stimulus source has not been identified as prey. A significantly greater response to prey chemicals than pungency controls permits the inference of chemosensory discrimination among the stimuli.

Paper towels and water bowls were removed from cages immediately before testing and were replaced immediately after testing each day. Trials were conducted from 13 : 30 to 16 : 30 hr CST with a minimum intertrial interval of 60 min. Before beginning data collection each day, the experimenter carefully removed the tops from all cages to avoid vibrations. To start a trial, the experimenter slowly approached a lizard's home cage and positioned the cotton swab 0.5–1 cm anterior to the lizard's snout. The number of tongue-flicks in the 60 sec starting with the first tongue-flick were recorded unless the lizard bit the swab. If the lizard bit the swab, the number of tongue-flicks emitted to that time and the latency from first tongue-flick to bite were recorded. If a lizard failed to tongue-flick the swab within 30 sec, the experimenter brought the swab into brief, gentle contact with the anterior labial scales, which often induces tongue-flicking. If the lizard tongue-flicked within the 30 sec after contact with the swab, its data were recorded as above. If it did not tongue-flick within the next 30 sec, zero tongue-flicks were recorded.

In the experiment on chemosensory response to animal prey and potential plant food, responses of each lizard to external cricket stimuli, romaine lettuce, cologne, and deionized water were tested in a randomized blocks design. Trial sequences for each individual were randomized. Nineteen individuals responded in all conditions and two more were discarded because they repeatedly fled. In the experiment on effects of surface versus internal prey chemicals on tongue-flicking and biting attacks, responses by 15 individuals (14 also used in experiment 1) to cricket surface and internal stimuli were tested in a randomized blocks

design. Eight individuals were tested first with internal and seven with surface stimuli.

For both experiments the data analyzed were number of tongue-flicks per trial, number of individuals that bit swabs, and a derived variable that combines tongue-flicking and biting, the tongue-flick attack score for repeated measures designs [TFAS(R)] (Cooper and Burghardt, 1990b). If a lizard does not bite the swab, its TFAS(R) is the number of tongue-flicks. If the lizard bites, TFAS(R) is the greatest number of tongue-flicks by that individual in any one condition plus (60—latency to bite in seconds). TFAS(R) combines chemosensory investigation as indicated by tongue-flicking with biting, a feeding attack, to give a composite measure of response strength. Biting is weighted more heavily than any number of tongue-flicks because it is a direct predatory response, and bites at shorter latency are given heavier weight than bites at longer latencies.

Due to the heterogeneity of variance and nonnormality, data were analyzed nonparametrically. For experiment 1 we used Friedman two-way analysis of variance to analyze tongue-flicks and TFAS(R) and sign tests to analyze bite data. Due to low expected frequencies of biting, statistical comparisons were restricted to tests of differences in number of bites between the prey stimuli and each control stimulus. Significant main effects were followed by paired comparisons tests as in Zar (1995). For experiment 2, tongue-flicks and TFAS(R) were analyzed by the Wilcoxon matched-pairs signed ranks test and bite data by a McNemar's test for significance of changes (Zar, 1995). We tested the significance of difference in frequency of bites in response to cricket surface stimuli in the two experiments with McNemar's test. Alpha was 0.05 and significance tests were two-tailed with the exception of the bite comparisons, in which one-tailed tests are justified for differences between the food and control stimuli and for the greater predicted response to internal than surface prey cues. For bite data from experiment 1, reported probabilities are unadjusted for the number of tests conducted, but we evaluated significance by a sequential Bonferroni procedure (Wright, 1992).

RESULTS

Food Chemical Discrimination. Numbers of tongue-flicks, numbers of individuals biting swabs, and TFAS(R) were all substantially greater to cricket stimuli than to any of the controls. Numbers of tongue-flicks (Figure 1) differed significantly among stimuli ($\chi^2 = 23.51$; $df = 3, 27$; $P < 0.001$). Comparisons among pairs of stimulus means showed that cricket stimuli elicited significantly more tongue-flicks than did romaine lettuce, cologne, or deionized water ($P < 0.001$ each). Numbers of tongue-flicks ranged widely (3–24) in response to cricket stimuli, the smallest numbers being for lizards that bit the swab rapidly. Mean tongue-flick rates in response to the three nonfood stimuli were strik-

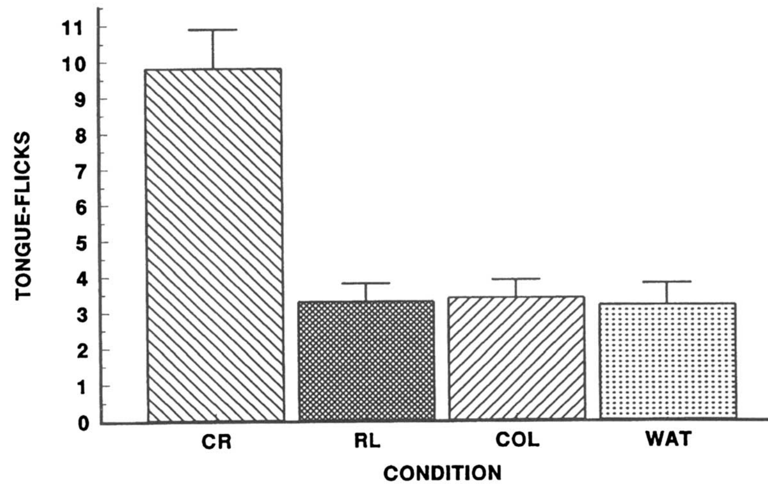

FIG. 1. Mean tongue-flicks in 60 sec by 19 *Scincella lateralis* directed to cotton swabs bearing chemical stimuli from cricket (CR), romaine lettuce (RL), cologne (COL), and deionized water (WAT). Error bars represent 1.0 SE.

ingly similar (Figure 1), as were the ranges (1–7 for romaine lettuce, 1–10 for cologne, and 1–8 for deionized water), and did not differ significantly ($P > 0.10$ each).

Biting was restricted to the cricket stimuli. Because six individuals bit swabs bearing cricket stimuli, biting was significantly more frequent in response to cricket stimuli than to each of the other three stimuli ($P < 0.016$ each). Among the individuals that bit, mean latency ± 1.0 SE was 5.83 ± 0.79, with range 3–8. No lizards bit without first tongue-flicking.

The greatest TFAS(R) (Figure 2) for each of the 19 individuals was to cricket stimuli. The main stimulus effect was highly significant ($\chi^2 = 34.64$; $df = 3, 27$; $P \ll 0.001$). TFAS(R), which had a range of 5–63, was significantly higher to cricket stimuli than to any of the controls ($P < 0.001$ each). In the absence of bites, TFAS(R) for each control condition was identical to number of tongue-flicks. Thus, TFAS(R) was remarkably similar among the other three test stimuli.

Internal Versus Surface Cues. Mean numbers of tongue-flicks elicited by both surface and internal cricket stimuli were similar to those observed in experiment one (Table 1). Numbers of tongue-flicks performed in response to internal and surface cricket stimuli did not differ significantly ($T = 50$, $N = 15$, $P > 0.10$) for all individuals tested. Means and ranges were very similar, with slightly higher variability in response to internal stimuli (Table 1). When the analysis was restricted to the individuals that did not bite in either condition, the number

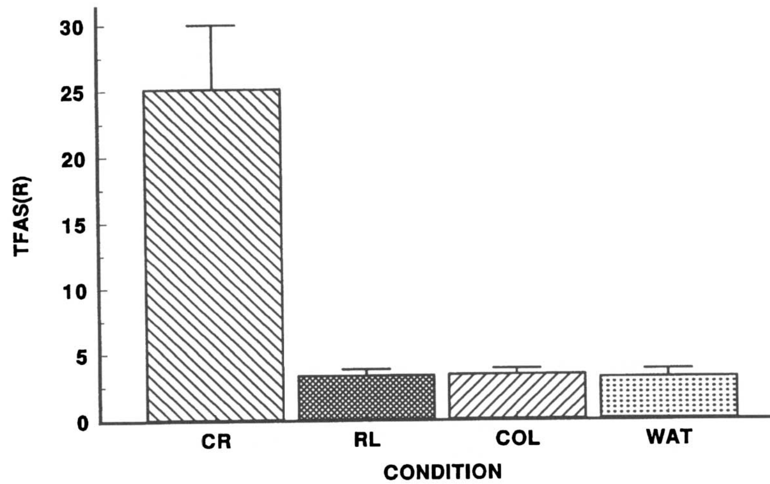

FIG. 2. Mean tongue-flick attack scores for 19 *Scincella lateralis* responding to chemical stimuli from cricket (CR), romaine lettuce (RL), cologne (COL), and deionized water (WAT). Error bars represent 1.0 SE.

of tongue-flicks was significantly greater in response to internal cues ($T = 0$, $N = 6$, $P < 0.05$).

A significantly greater number of individuals bit swabs bearing internal than external cricket stimuli ($P < 0.016$). For the nine individuals that bit the internal cricket stimulus, latency to bite was 8.33 ± 2.13 sec (range 2–12 sec). For the two

TABLE 1. TONGUE-FLICKS (TF) IN 60 SECONDS AND TONGUE-FLICK ATTACK SCORES [TFAS(R)] BY *Scincella lateralis* IN RESPONSE TO SWABS BEARING SURFACE OR INTERNAL CRICKET CHEMICALS

	$\bar{X}$	SE	Range
All individuals (N = 19)			
TF			
Surface	8.4	1.2	3–22
Internal	9.3	1.7	1–22
TFAS(R)			
Surface	15.8	4.8	4–62
Internal	44.1	6.8	8–80
Individuals that did not bite (N = 6)			
TF			
Surface	6.3	1.0	4–10
Internal	14.5	2.0	8–22

that bit the surface stimulus latency was 5.0 ± 3.0 sec (range 2–8 sec). No lizards bit without first tongue-flicking. Although fewer individuals bit in response to the cricket surface stimuli in experiment 2 than in experiment 1, this difference was not significant for individuals that participated in both experiments ($P > 0.10$).

Counter to any tendency for waning response with repeated presentation, mean response to internal stimuli in experiment 2 was more than 50% greater than that to surface stimuli in experiment 1. More importantly, mean TFAS(R) was nearly three times greater in response to internal than surface stimuli in experiment 2. This was a consistent effect, TFAS(R) being greater in response to internal stimuli than to surface stimuli in 14 of 15 individuals. The remaining individual bit swabs in both conditions, but at greater latency in the internal condition. TFAS(R) was significantly greater in response to internal than external cricket stimuli ($T = 8$, $N = 15$, $P < 0.005$).

DISCUSSION

Responses to Plant and Animal Chemical Stimuli. There was no discriminatory response to chemical stimuli from romaine lettuce, as indicated by the lack of significant differences in tongue-flick rates and TFAS(R) between the romaine lettuce and the control and by absence of biting in response to romaine lettuce cues. This contrasts markedly with strong responses by several herbivorous lizards to chemical cues from romaine lettuce and other plants (Cooper and Alberts, 1990; Cooper, unpublished data). Because the statistical power for this sort of experiment with a sample size of 19 is >0.95 (Winer, 1962), we can be confident that a type 2 error did not occur, i.e., that no undetected lingually mediated discrimination occurred. Several informal trials further revealed no strong responses to stimuli from red grape, banana, or strawberry.

If chemosensory response to food is adaptively related to diet, insectivorous lizards such as *S. lateralis* that rarely, if ever, voluntarily consume plants (Lewis, 1951; Hamilton and Pollack, 1961; Brooks, 1963) are expected to lack a strong response to chemical stimuli from palatable plants. Our findings are consistent with the hypothesis that responsiveness to plant chemicals is a derived trait in taxa that evolved herbivory or omnivory rather than a plesiomorphic condition retained from insectivorous ancestors. Comparative data on additional herbivores and insectivores are needed for a phylogenetic analysis to test this hypothesis.

In contrast to its lack of response to plant stimuli, *S. lateralis* avidly tongue-flicked and attacked stimuli from crickets. Prey chemical discrimination is demonstrated by the significantly greater numbers of tongue-flicks, bites, and TFAS(R) in response to cricket stimuli than to all of the other stimuli in experiment one. These findings corroborate the elevated tongue-flicking in response to prey (cockroach) extract on a pipe cleaner left in the home cage in the absence of visual prey cues reported by Nicoletto (1985). Like all other actively forag-

ing lizards studied to date (Cooper, 1994a,b, 1995, 1997, 1998b), *S. lateralis* is capable of lingually mediated discrimination between chemical cues from animal prey and control substances.

Surface Versus Internal Cues. The lizards responded much more strongly to internal than to surface prey chemicals, as shown by the much greater biting frequency and TFAS(R). For the entire data set, numbers of tongue-flicks in response to internal and external stimuli did not differ because biting precluded further tongue-flicking. For individuals that bit in one condition but not in the other, there was often much more time for tongue-flicking in the condition that did not evoke biting. For individuals that did not bite in either condition, the significantly greater number of tongue-flicks in response to internal prey cues corroborates the findings for biting and TFAS(R).

Internal chemical stimuli seem less appropriate for studies of the ability of lizards to detect and identify prey than external stimuli, but are relevant to responses to food that has been bitten or has been injured in a manner exposing internal chemicals. This may account for the strong chemosensory and ingestive responses of brown tree snakes (*Boiga irregularis*) to mammalian blood (Chiszar et al., 1993). Stronger responses to internal than surface prey chemicals might reflect a greater concentration of similar effective substances but is likely due to a combination of qualitative and quantitative differences in the mixture of surface and internal chemicals. Nevertheless, the strong responses to both sources of chemical prey cues indicate that both are treated as indicators of food. Information based on either source is probably adequate for qualitative comparative studies examining presence/absence of prey chemical discrimination. However, the pronounced difference in response strength to internal and surface chemicals shows that a consistent method of stimulus preparation must be used for interspecific comparisons when using continuous response variables.

Differences among swabbed surface stimuli and extracts or directly swabbed samples containing internal chemicals might cause discrepant results among studies. Extracts have the advantages of repeatability and facilitating preparation of purified samples. The primary advantage of swabbing surface stimuli is realism in that the same set of chemicals that may be contacted by a squamate's tongue is sampled in situ. A major consideration for lizard studies is that almost the entire comparative data set is based on raw surface stimuli.

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EVALUATION OF DIMBOA ANALOGS AS ANTIFEEDANTS AND ANTIBIOTICS TOWARDS THE APHID *Sitobion avenae* IN ARTIFICIAL DIETS

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Abstract—A total of 25 compounds including benzoxazinones, benzoxazolinones, and *N*-glyoxylamide derivatives were tested as antifeedants and antibiotics towards the aphid *Sitobion avenae* in diet bioassays. The antifeedant and mortality indexes increased with the presence of electron-donating groups in the 7 position of the benzoxazinone moiety, the replacement of the oxygen atom by sulfur in the heterocyclic ring, the presence of a hemiacetal instead of an acetal at C-2 of the benzoxazine moiety (and hence the possibility of ring opening), and the presence of a hydroxyl group at C-4 of the benzoxazine moiety (hydroxamic acid) instead of a hydrogen atom (lactam). The results support earlier hypotheses on the chemical bases for the mode of action of these compounds.

Key Words—Aphids, *Sitobion avenae*, feeding deterrents, antibiotics, DIMBOA.

INTRODUCTION

2-*β*-*O*-D-Glucopyranosides containing a 2,4-dihydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one moiety (**I**, Figure 1) are found mainly in the family Poaceae (Niemeyer, 1988) and confer the plant resistance against bacteria, fungi, and insects, including aphids (Niemeyer and Pérez, 1995). The naturally present

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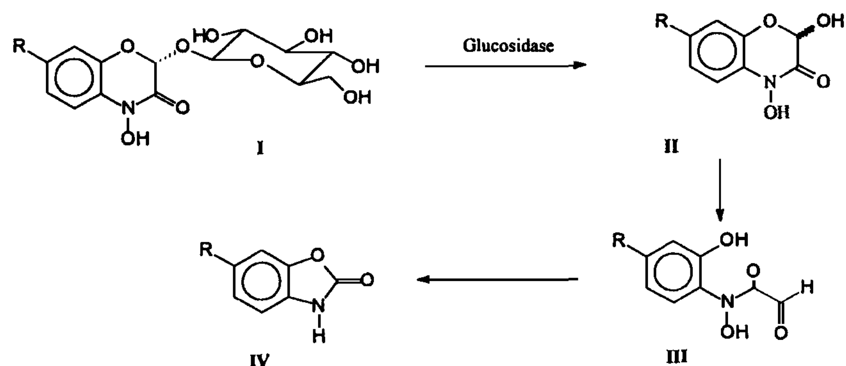

FIG. 1. **I:** 2-β-D-Glucopyranoside of a (2*R*)-2,4-dihydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one; **II:** a 2,4-dihydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one, the aglucone, liberated upon tissue damage by the action of a β-glucosidase. DIMBOA (R = OCH_3), TRIBOA (R = OH), DIBOA (R = H). **III:** the *N*-(2-hydroxyphenyl)-glyoxylo-hydroxamic acid produced in solution by the opening of the hemiacetal moiety; **IV:** the benzoxazolin-2(3*H*)-one produced as decomposition product of **II.**

glucosides are hydrolyzed by *endo*-β-glucosidases liberated following plant damage (Hofman and Hofmanova, 1969), producing the more toxic aglucones (**II**, Figure 1). In solution, these aglucones decompose through a mechanism involving the opening of the hemiacetal moiety to produce a *N*-(2-hydroxyphenyl)glyoxylohydroxamic acid (**III**, Figure 1), which further yields a benzoxazolin-2(3*H*)-one (**IV**, Figure 1) (Smissman et al., 1972; Bravo and Niemeyer, 1985). Recently the synthetic approaches, both to acetal glucosides and aglucones, have been reviewed (Sicker et al., 1997).

The main hydroxamic acid aglucone in wheat and maize extracts is 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIMBOA), while in rye it is 2-hydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIBOA). Trace amounts of 2,4,7-trihydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one (TRIBOA) are found in maize (Woodward et al., 1979).

The effect of DIMBOA on aphids has been studied in detail. An antibiotic effect has been described. Thus, inverse relationships were found between the DIMBOA level in maize and infestation by *Rhopalosiphum maidis* Fitch (Long et al., 1977); a similar situation was also reported for wheat and *Metopolophium dirhodum* Walk. (Argandoña et al., 1980), *Schizaphis graminum* Rond. (Argandoña et al., 1981), and *Sitobion avenae* Fabr. (Bohidar et al., 1986). When added to artificial diets, DIMBOA decreased survival, weight gain, and reproduction of several cereal aphid species (Argandoña et al., 1980; Niemeyer et al., 1989; Givovich and Niemeyer, 1995). On the other hand, an antifeeding

effect has also been described. Thus, negative relationships were found between the DIMBOA content of wheat plants and the number of aphids feeding on them, and their honeydew production (Niemeyer et al., 1989); in choice tests, aphids preferred to settle and took longer time to attain a sustained phloem ingestion on wheat plants with lower DIMBOA content (Nicol et al., 1992; Givovich and Niemeyer, 1991); when added to artificial diets, DIMBOA inhibited aphid feeding (Argandoña et al., 1983).

The present work evaluates the effect of structurally related 2*H*-1,4-benzoxazin-3(4*H*)-ones, their decomposition products, and some open chain analogs on the aphid *S. avenae*, in choice and antibiosis tests employing artificial diets.

METHODS AND MATERIALS

Aphids. To start the cultures individuals of *S. avenae* were collected randomly in grass fields near the Laboratorio de Química Ecológica in Santiago, Chile, and the multiclonal cultures were maintained on oat (*Avena sativa* L. cv. Nehuén) for at least five generations in a greenhouse at 18–22°C, 18L : 6D photoperiod, and 50–70% relative humidity. Individuals used in the bioassays were chosen randomly from the culture, subject to the conditions that they had recently molted to adults and were in an active food site-searching mood.

Compounds. Melting points were determined in a Boetius micro hot-stage apparatus and are corrected. The NMR spectra were recorded on a Varian Gemini 200 spectrometer at 199.975 MHz for ^{1}H and at 50.289 MHz for ^{13}C in either $CDCl_3$ or DMSO-d_6 as solvent and hexamethyldisiloxane as internal standard. The IR spectra were obtained on an ATT Mattson spectrometer in potassium bromide. Mass spectra were recorded on a Finnigan MAT 212 spectrometer (70 eV EI ionization, source temperature 200°C). 2,4-Dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one (**1**, DIMBOA), was isolated from *Zea mays* L. cv. T55s according to the protocol published by Hartenstein et al. (1992); and benzoxazolin-2(3*H*)-one (**18**) was of commercial grade (Aldrich). The following compounds were synthesized as reported: 2-methoxy-4-hydroxy-2*H*-1,4-benzothiazin-3(4*H*)-one (**2**) (Sicker et al., 1994); 2,4-dihydroxy-2*H*-1,4-benzothiazin-3(4*H*)-one (**3**) (Sicker et al., 1994); 2-methoxy-2*H*-1,4-benzothiazin-3(4*H*)-one (**4**) (Zahn, 1923); 2,4,7-trihydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one (**5**) (Kluge et al., 1995); 2-hydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one (**6**) (Sicker and Hartenstein, 1993); 2,4-dihydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one (**7**) (Sicker et al., 1989); 2*H*-1,4-benzothiazin-3(4*H*)-one (**8**) (Sicker et al., 1994); 2-methoxy-4,7-dihydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one (**9**) (Kluge et al., 1995); 2,7-dimethoxy-4-hydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one (**10**) (Atkinson et al., 1991); 2-hydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one (**11**)

(Sicker and Hartenstein, 1993); 4-hydroxy-2*H*-1,4-benzothiazin-3(4*H*)-one (**12**) (Sicker et al., 1994); 2*H*-1,4-benzoxazin-3(4*H*)-one (**13**) (Honkanen and Virtanen, 1960); 2-methoxy-4-hydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one (**14**) (Atkinson et al., 1991); 4-hydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one (**15**) (Hartenstein and Sicker, 1994); 2-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one (**16**) (Atkinson et al., 1991); 6-methoxy-benzoxazolin-3(3*H*)-one (**17**) (Sicker, 1989); and 6-hydroxy-benzoxazolin-2(3*H*)-one (**19**) (Wieland et al., 1999). Other compounds were synthesized as described below.

N-*(2′,4′-Dimethoxyphenyl)-2,2-diethoxyacetamide (20).* A mixture of 2,4-dimethoxyaniline (15.3 g; 0.1 mol), and 2,2-diethoxyethanoic acid (14.8 g; 0.1 mol) was refluxed in toluene for 4 hr in a Dean Stark apparatus. The solution was cooled, washed with water (2 × 250 ml), dried ($MgSO_4$), and evaporated under vacuum to give a colorless oil that crystallized in the freezer overnight to produce 22 g (77%) of **20** as colorless crystals (mp 36–37°C). IR: 1531, 1263 cm^{-1}; ^1H NMR (DMSO-d_6) δ: 1.21 (t, 6H, 2 × CH_3, J = 7.2 Hz), 3.65 (q, 4H, 2 × CH_2), 3.76 (s, 3H, OCH_3), 3.86 (s, 3H, OCH_3), 4.95 (s, 1H, CH), 6.53 (dd, 1H, $H_{5'}$, $^4J_{5',3'}$ = 2.7 Hz, $^3J_{5',6'}$ = 8.8 Hz), 6.67 (d, 1H, $H_{3'}$, $^4J_{5',3'}$ = 2.7 Hz), 7.98 (d, 1H, $H_{6'}$, $^3J_{5',6'}$ = 8.8 Hz), 8.85 (s, 1H, NH); ^{13}C NMR (DMSO) δ: 15.3 (CH_3), 55.6 (OCH_3), 56.3 (OCH_3), 62.4 (OCH_2), 98.6 (CH), 99.1 (C-3′), 104.4 (C-5′), 119.9 (C-6′), 121.1 (C-1′), 150.3 (C-2′), 156.9 (C-4′), 165.3 (CO); MS: m/z 283 (M^+, 19), 238 (2), 209 (6), 152 (18), 103 (100).

N-*(2′,4′-Dimethoxyphenyl)glyoxylamide (21).* To a cooled (−25°C) solution of glyoxylic acid monohydrate (2.76 g; 0.03 mol) in THF (20 ml) was added a solution of dicyclohexylcarbodiimide (8.25 g; 0.04 mol) in THF (30 ml). When a white precipitate began to form, a cooled solution of 2,4-dimethoxyaniline (2.29 g; 0.015 mol) dissolved in THF (10 ml) was added portionwise. After 1 hr, the solid formed was filtered off and the organic fraction evaporated under vacuum. The product obtained was chromatographed (silicagel 60; toluene–ethyl acetate 2 : 1 v/v) to give 0.74 g (23.4%) of **21** as white solid (mp 212–214°C). IR: 1504, 1547 cm^{-1}; ^1H NMR ($CDCl_3$) δ: 3.82 (s, 3H, OCH_3), 3.89 (s, 3H, OCH_3), 6.4 (dd, 1H, $H_{5'}$, $^3J_{5',6'}$ = 8.8 Hz, $^4J_{5',3'}$ = 2.7 Hz), 6.51 (d, 1H, $H_{3'}$, $^4J_{5',3'}$ = 2.7 Hz), 8.07 (d, 1H, $H_{6'}$, $^3J_{5',6'}$ = 8.8 Hz), 9.01 (s, 1H, NH); ^{13}C NMR ($CDCl_3$) δ: 56.0 (OCH_3), 56.3 (OCH_3, 99.2 (C-3′), 104.4 (C-5′), 119.6 (C-1′), 121.3 (C-6′), 150.6 (C-2′), 157.5 (C-4′), 158.1 (CO), 189.1 (CHO); MS: m/z 209 (M^+, 100), 180 (37), 165 (24), 152 (89), 138 (61).

N-*(2′,4′-Dimethoxyphenyl)-2,2-dichloroacetamide (22).* A solution of 2,4-dimethoxyaniline (1.53 g; 0.01 mol) and 4-dimethylaminopyridine (1.50 g; 0.012 mol) in chloroform (50 ml) was cooled to 0°C by means of an ice-water bath. The solution was stirred and treated with 2,2-dichloroacetyl chloride (1.47 g; 0.01 mol), which was added in portions over 30 min, keeping the temperature between 2 and 5°C. The course of the reaction was monitored by TLC (toluene–ethyl acetate 1 : 2 v/v). When the reaction was complete, the solution was washed with

diluted HCl (2 × 60 ml). The organic layer was dried ($MgSO_4$) and evaporated under vacuum to give a white solid, which was recrystallized from cyclohexane to give 2.26 g (86%) of **22** as colorless crystals (mp 110–111°C). IR: 804, 836, 1036, 1124, 1554 cm^{-1}, ^1H NMR ($CDCl_3$) δ: 3.80 (s, 3H, OCH_3), 3.89 (s, 3H, OCH_3), 6.04 (s, 1H, CH), 6.46–6.52 (m, 2H, $H_{3'}$ + $H_{5'}$), 8.17 (d, 1H, $H_{6'}$, $^3J_{5',6'}$ = 9 Hz), 8.69 (s, 1H, NH); ^{13}C NMR ($CDCl_3$) δ: 56.0 (OCH_3), 56.4 (OCH_3), 67.5 ($CHCl_2$), 99.2 (C_3), 104.3 (C_5), 120.2 (C_6), 121.1 (C_1), 150.3 (C_2), 157.98 (C_4), 161.6 (CO); MS: m/z 263 (M^+, 73), 180 (34), 152 (100), 138 (14), 124 (24).

N-*(2′,4′-Dimethoxyphenyl)-2-ethoxyglyoxylamide (23).* A solution of 2,4-dimethoxyaniline (1.53 g; 0.01 mol) and 4-(dimethylamino)-pyridine (1.50 g; 0.012 mol) in chloroform (50 ml) was cooled to 0°C and under stirring was treated dropwise with ethyl oxalyl chloride (1.36 g; 0.01 mol) keeping the temperature between 2 and 5°C. After 4 hr, the solution was washed with diluted HCl (2 × 60 ml) and the organic layer dried ($MgSO_4$) and evaporated under vacuum to give a white solid, which was recrystallized from cyclohexane to give 1.83 g (72%) of **23** as colorless crystals (mp 145–147°C). IR: 830, 1266, 1542, 1732 cm^{-1}; ^1H NMR ($CDCl_3$) δ: 1.42 (t, 3H, CH_3, $^3J_{1'',2''}$ = 7.2 Hz), 3.79 (s, 3H, OCH_3), 3.88 (s, 3H, OCH_3), 4.41 (q, 2H, CH_2, $^3J_{1'',2''}$ = 7.2 Hz), 6.49 (m, 2H, $H_{3'}$ + $H_{5'}$), 8.31 (d, 1H, $H_{6'}$ $^3J_{5',6'}$ = 9.6 Hz), 9.31 (s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ: 14.5 (CH_3), 56.0 (OCH_3), 56.3 (OCH_3), 63.8 (OCH_2), 99.2 (C-3), 104.3 (C-5), 120.3 (C-1), 121.2 (C-6), 150.3 (C-2), 153.8 (CO), 157.9 (C-4), 161.5 (CO); MS: m/z 253 (M^+, 78), 179 (100), 164 (21), 152 (66), 122 (61).

N-*(2′,4′-Dimethoxyphenyl)-2-hydroxyacetamide (24).* A mixture of 2,4-dimethoxyaniline (3.06 g; 0.02 mol), glycolic acid (1.52 g; 0.02 mol), and toluene (90 ml) was refluxed for 5 hr in a Dean Stark apparatus. The solution was cooled, and the white solid formed was filtered and recrystallized from cyclohexane to give 3.8 g (90%) of **24** as colorless crystals (mp 132–134°C). IR: 818, 1033, 1128, 1561, 3185 cm^{-1}; ^1H NMR (DMSO-d_6) δ: 3.76 (s, 3H, OCH_3), 3.86 (s, 3H, OCH_3), 6.05 (s, 1H, OH), 6.52 (dd, 1H, $H_{5'}$, $^3J_{5',6'}$ = 8.8 Hz, $^4J_{3',5'}$ = 2.7 Hz), 6.67 (d, 1H, $H_{3'}$, $^4J_{3',5'}$ = 2.7 Hz), 8.10 (d, 1H, $H_{6'}$, $^3J_{5',6'}$ = 8.8 Hz), 8.96 (s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ: 55.6 (OCH_3), 56.2 (OCH_3), 61.9 (CH_2OH), 99.0 (C-3′), 104.4 (C-5′), 120.3 (C-6′), 120.5 (C-1′), 149.7 (C-2′), 156.4 (C-4′), 164.9 (CO); MS: m/z 211 (M^+, 56), 153 (64), 138 (46), 124 (16), 51 (100).

N-*(2′,4′-Dimethoxyphenyl)-acetamide (25).* A solution of 2,4-dihydroxyaniline (1.53 g; 0.01 mol) and 4-dimethylaminopyridine (1.50 g; 0.012 mol) in chloroform (50 ml) was cooled to 0°C by means of an ice-water bath. The solution was stirred and treated with acetic anhydride (1.22 g; 0.012 mol), which was added in portions over 30 min, keeping the temperature between 2 and 10°C. The course of the reaction was monitored by TLC (toluene–ethyl acetate 1 : 2 v/v. When the reaction was complete, the solution was washed with diluted HCl

(2 × 60 ml). The organic layer was dried ($MgSO_4$) and evaporated under vacuum to give a colorless solid, which was recrystallized from cyclohexane to give 0.8 g (41%) of **25** as colorless crystals (mp 115–116°C). IR: 800, 939, 1207, 1024, 1539 cm^{-1}; ^1H NMR ($CDCl_3$) δ: 2.16 (s, 3H, $COCH_3$), 3.78 (s, 3H, OCH_3), 3.83 (s, 3H, OCH_3), 6.45 (m, 2H, $H_{3'}$ + $H_{5'}$), 7.53 (s, 1H, NH), 8.22 (d, 1H, $H_{6'}$, $^3J_{5',6'}$ = 9.5 Hz); ^{13}C NMR ($CDCl_3$) δ: 25.1 (CH_3), 55.9 (OCH_3), 56.1 (OCH_3), 99.0 (C-3), 104.2 (C-5), 121.3 (C-6), 121.8 (C-1), 149.7 (C-2), 156.9 (C-4), 168.4 (CO); MS: m/z 195 (M^+, 36), 153 (22), 138 (32), 122 (11), 42 (100).

Choice Tests. To determine the effect of the compounds on the feeding activity of aphids, 10 aphids were confined in a horizontally placed Plexiglas cylinder (3.0 cm long, 2.5 cm ID). To one end of the cylinder a sachet made of two Parafilm M membranes (American Can Co.) and containing 200 μL of a solution consisting of sucrose (25% w/v) and a small amount of dimethylsulfoxide (DMSO, 0.05% w/v) was attached. This side was named "control" (C). To the other end of the cylinder, a similar sachet containing the compound to be tested (0.5 mg/ml), was attached [treatment (T)]. The aphids were allowed to choose between the diets in a dark room maintained at 18–22°C and 50–70% relative humidity. After 18 hr, the number of feeding aphids in the treatment side (T) and the number of feeding aphids on the control side (C) were recorded. Feeding was judged by the immobility of the aphids and their antennae pointing backwards. An antifeedant index was defined as $(C - T)/(C + T)$ (Powell et al., 1997) in order to estimate the repellent or stimulant effects of the compounds on feeding activity. The antifeedant index may vary between 1 and -1, where 1 means maximum antifeedant activity; 0 no activity, and -1 maximum phagostimulant activity. The experiment was replicated 25 times for each compound tested.

Mortality Test. Ten aphids were confined in a vertically placed Plexiglas cylinder (3.0 cm high, 2.5 cm ID). One end of the cylinder was closed with a plastic net, the other with a sachet made of two Parafilm M membranes, containing 200 μl of a sucrose solution (25% w/v), a small amount of DMSO (0.05% w/v), and the compound to be tested at a concentration of 2 mM. Control cylinders were prepared in the same way but contained only 200 μl of sucrose solution (25% w/v) and DMSO (0.05% w/v). The cylinders containing the aphids were maintained at 18–22°C, 18L : 6D photoperiod and 50–70% relative humidity. After 89 hr, the number of dead aphids in the treatment and in the paired control cylinders were recorded. When aphid reproduction occurred, the progeny were not taken into account. A mortality index was defined as the mean of the number of dead aphids in the treatment minus that of the paired control. Twenty-five replicates were performed for each compound tested.

Statistical Analysis. Comparisons of activities within sets of chemically related compounds (the sets are defined in the Results section) were performed

with a nonparametric one-way ANOVA (Kruskal-Wallis test) followed by the Bonferroni correction. When significant differences between activities were found, a posteriori multiple comparisons were performed (Siegel and Castellan, 1988).

RESULTS

Bioassays. In the choice bioassay, the mean number of dead aphids at the end of the experiments was 0.078 ± 0.00006 ($N = 625$), and the mean number of aphids neither dead nor feeding on the diets at the end of the experiment was 2.14 ± 0.00025 ($N = 625$). In the antibiosis bioassay, the mean number of dead aphids in the control cylinder at the end of the experiments was 1.25 ± 0.0022 ($N = 625$), and the mean number of aphids neither dead nor feeding in the diet at the end of the experiment was 2.21 ± 0.0022 ($N = 625$) in the control cylinders and 2.16 ± 0.00045 ($N = 625$) in the treatment cylinders.

Activities. The antifeedant and the mortality indexes are tabulated as average values followed by their standard errors in Table 1. Five sets of compounds were defined that addressed different chemical questions through pairwise comparisons: (1) nature of the substituent at C-7 (pairs **7–5**, **7–1**, **14–9**, and **14–10**); (2) substitution of oxygen by sulfur at position 1 of the heterocyclic ring (pairs **7–3**, **13–8**, **14–2**, **15–12**, and **16–4**); (3) hydroxyl or methoxyl group at C-2, i.e., hemiacetal or acetal (pairs **9–5**, **10–1**, **14–7**, and **16–6**); (4) hydroxyl or hydrogen on nitrogen, i.e., hydroxamic acid or lactam (pairs **4–2**, **11–1**, **16–14**, **6–7**, **8–12**, and **13–15**); and (5) benzoxazinone- or benzoxazolinone-type compounds (pairs **1–17**, **5–19**, and **7–18**).

Within the benzoxazinone series, the antifeedant index consistently showed a tendency to increase (an asterisk denotes a significant difference i.e., $P < 0.05$) upon substitution with electron-donating groups at C-7 (**7** < **5**, **7** < **1***, **14** < **9***, and **14** < **10**), replacement of the heterocyclic oxygen atom by sulfur (**7** < **3***, **13** < **8***, **14** < **2***, **15** < **12***, and **16** < **4***), and the presence of a hydroxyl group instead of a methoxy group as substituent at C-2 (**9** < **5***, **10** < **1***, **14** < **7***, and **16** < **6***). The replacement of hydrogen by hydroxyl on the heterocyclic nitrogen atom tended to increase the antifeedant index in some cases (**4** < **2***, **11** < **1***, and **16** < **14**) and to decrease it in others (**6** > **7**, **8** > **12***, and **13** > **15**). The mortality index showed a tendency to increase upon substitution with electron-donating groups at C-7 (**7** < **1**, **14** < **9***, and **14** < **10**; however, **7** > **5***), replacement of the heterocyclic oxygen atom by sulfur (**7** < **3**, **13** < **8***, **14** < **2***, **15** < **12**, and **16** < **4***), and the presence of a hydroxyl group instead of a methoxy group as substituent as C-2 (**10** < **1***, **14** < **7***, and **16** < **6***; however, **9** > **5**). The replacement of hydrogen by hydroxyl on the heterocyclic nitrogen atom tended to increase the antifeedant index in some cases (**4** < **2***, **6** < **7***, **11** < **1***, and **16** < **14***) and to decrease it in others (**8** > **12*** and **13** > **15**).

TABLE 1. ACTIVITY OF COMPOUNDS TESTED

Compound	X	R^2	R^4	R^7	Antifeedant index[a]	Mortality[a]
1	O	OH	OH	OCH_3	0.547 ± 0.027	5.52 ± 0.14
2	S	OCH_3	OH	H	0.462 ± 0.024	5.09 ± 0.11
3	S	OH	OH	H	0.418 ± 0.030	5.62 ± 0.09
4	S	OCH_3	H	H	0.375 ± 0.025	1.78 ± 0.20
5	O	OH	OH	OH	0.347 ± 0.020	1.04 ± 0.15
6	O	OH	H	H	0.294 ± 0.022	0.88 ± 0.12
7	O	OH	OH	H	0.271 ± 0.024	5.46 ± 0.17
8	S	H	H	H	0.232 ± 0.021	5.34 ± 0.15
9	O	OCH_3	OH	OH	0.204 ± 0.028	1.24 ± 0.16
10	O	OCH_3	OH	OCH_3	0.124 ± 0.032	0.52 ± 0.16
11	O	OH	H	OCH_3	0.114 ± 0.031	1.52 ± 0.16
12	S	H	OH	H	0.087 ± 0.028	0.28 ± 0.07
13	O	H	H	H	0.041 ± 0.024	0.72 ± 0.09
14	O	OCH_3	OH	H	0.018 ± 0.028	0.48 ± 0.10
15	O	H	OH	H	−0.035 ± 0.023	0.19 ± 0.14
16	O	OCH_3	H	H	−0.103 ± 0.030	0.03 ± 0.11
		R^6				
17		OCH_3			0.435 ± 0.018	0.25 ± 0.16
18		H			0.257 ± 0.023	0.48 ± 0.07
19		OH			0.136 ± 0.028	1.04 ± 0.10
		R				
20		$CH(OEt)_2$			0.497 ± 0.024	0.96 ± 0.13
21		CHO			0.451 ± 0.018	1.62 ± 0.22
22		$CHCl_2$			0.125 ± 0.027	0.63 ± 0.05
23		COOEt			−0.218 ± 0.024	0.57 ± 0.04
24		CH_2OH			−0.253 ± 0.026	0.62 ± 0.04
25		CH_3			−0.407 ± 0.028	0.34 ± 0.03

[a]Mean ± standard error.

Benzoxazinones tended to be more active than the respective benzoxazolinones to which they decompose [**1** > **17***, **5** > **19***, and **7** > **18**). Within the open-chain analogs, higher antifeedant indexes were associated with the presence of electronegative substituents at the amidic carbonyl group, the trend being less clear in the mortality indexes.

DISCUSSION

Bioassay conditions represent, in general, a stress to the test individuals. Particularly in the antibiosis test, which used a minimal diet and lasted 89 hr, aphids may have been significantly stressed. However, the low mean mortality observed in the control experiments indicated that this stress did not compromise the survival of the test aphids. Moreover, the low number of aphids found neither dead nor feeding and the similarity between those found in the test and control experiments validated the bioassays for comparative purposes.

A chemical mechanism to account for the biological activities of hydroxamic acids containing the 2,4-dihydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one moiety has been proposed in which the electrophilic groups in **II** and the derived compound **III** (Figure 1) react and modify nucleophilic targets in metabolically relevant molecules in the biological environment (Niemeyer and Pérez, 1987). As bases for this proposal, hydroxamic acids of structure **II** react with thiols via a redox reaction involving the hydroxamic nitrogen atom and addition reactions involving the aldehydic carbonyl group of intermediate **III** (Pérez and Niemeyer, 1985) and with amines via the aldehydic and the amidic carbonyl groups of intermediate **III** (Pérez and Niemeyer, 1985) and with amines via the aldehydic and the amidic carbonyl groups of intermediate **III** (Pérez and Niemeyer, 1989b). They also inhibit the enzymes papain (Pérez and Niemeyer, 1989a), α-chymotrypsin (Cuevas et al., 1990), and aphid cholinesterases (Cuevas and Niemeyer, 1993) by reaction with nucleophilic residues in them.

The redox reaction with thiols is blocked when hydroxamic acids are transformed into the corresponding amides, and the formation of **III** is more favorable in the case of amides as compared with hydroxamic acids (Atkinson et al., 1991) or is completely blocked when the hemiacetal is transformed into an acetal. Furthermore, the reactivity of the amidic carbonyl group in **III** may be enhanced by electron-withdrawing substituents on it. In accordance with this chemical description, substitution of hydroxyl by methoxyl at C-2 decreased activity, some lactams were less active than the corresponding hydroxamic acids, and the presence of an α,β-dicarbonyl moiety or electron-withdrawing groups at the amidic carbonyl group in the open-chain compounds produced comparatively higher antifeedant and mortality indexes.

The finding that substitution of oxygen by sulfur at position 1 of the het-

erocyclic ring and the incorporation of electron-donating groups at C-7 of the benzoxazinones increased the activity of compounds in both bioassays can not be explained on the basis of the mechanism presented above. Chemical studies with compounds such as **II**, which lacked the hydroxyl group at C-2, showed that the hydroxamic hydroxyl group was transformed by acylation into a better leaving group, which, upon departure, formed a highly electrophilic nitrenium ion-type intermediate (Hashimoto et al., 1979, 1991; Hashimoto and Shudo, 1996; Ishizaki et al., 1992). This electrophile has been shown to further react with biologically relevant molecules possessing nucleophilic sites. Formation of the electrophilic intermediate should be favored by electron-donating groups. In accordance with this chemical description, substitution of hydrogen by either hydroxyl or methoxyl in the aromatic ring and also substitution of oxygen by sulfur in the heterocyclic ring increased the antifeedant and mortality indexes.

Two comparisons can not be rationalized in the chemical terms described above: compound **5** showed significantly higher indexes than compound **12**, in spite of being a lactam with no possibility of ring opening, and compound **7** showed significantly higher mortality than compound **5**, in spite of lacking an electron-donating substituent in the aromatic ring. It is likely that other chemical interactions with biological substrates, such as hydrophilic–hydrophobic interactions, predominate in these cases.

The results presented indicate that the antifeedant and antibiotic activities of hydroxamic acid derivatives can not be rationalized in terms of a single mechanism accounting for the chemical reactivity of the compounds tested. This is not surprising since, most likely, taste and susceptibility to the toxic effects of xenobiotics in an aphid are complex phenomena in which more than one cascade of events leads to the same final effect. Consequently, different compounds may show the same final effect, albeit by affecting different pathways or different steps in a given pathway, presumably by different chemical mechanisms, depending on the structural features of the xenobiotic at hand.

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YEASTS REDUCE THE STIMULATORY EFFECT OF ACETATE ESTERS FROM APPLE ON THE GERMINATION OF *Botrytis cinerea* CONIDIA

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Abstract—The fungus, *Botrytis cinerea*, causes decay in apples in postharvest storage. Conidia of *B. cinerea* on polycarbonate membranes were incubated on filter paper disks saturated with water or suspensions of yeasts (2×10^6 CFU/filter) and then exposed for 24 hr at 22°C to 0–16 μl of ethyl, butyl, or hexyl acetates injected into the headspaces of 500-cc glass jars. Germination of conidia was increased by exposure to 4–16 μl of ethyl acetate compared to the no-ester controls. Conidia were stimulated to germinate by 4 μl of butyl acetate and 8 μl of hexyl acetate, but greater volumes were toxic to germination. The yeasts *Cryptococcus laurentii* and *Sporobolomyces roseus* were more effective at reducing the stimulatory effect than *Saccharomyces cerevisiae*. Acetate esters also stimulated germination of conidia on polycarbonate membranes on apples inside jars. *Cryptococcus laurentii* or *S. roseus* suppressed this effect more than *S. cerevisiae*. Germination of conidia on a membrane on water inside a 3.9-liter jar containing an apple was 2.5-fold greater than the no-apple control. Butyl acetate increased *Botrytis* infection of apple wounds. Butyl and hexyl acetate were detected by GLC in the headspace of Golden Delicious apples. Results indicated that some acetate esters produced by apple stimulated germination of *B. cinerea* conidia, and this effect was suppressed by yeasts.

Key Words—Yeasts, *Botrytis cinerea*, apple, volatiles, gray mold, biological control, fungal interaction, acetate esters.

INTRODUCTION

Apple fruit produce many different volatile compounds (Mattheis et al., 1991; Knee, 1993; Vishniac et al., 1997). Some of these have been shown to affect the germination and growth of fungi. For instance, benzaldehyde at 1250 μl per liter

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completely suppressed in vitro germination of *Botrytis cinerea* Pers.: Fr. conidia (Wilson et al., 1987), whereas ethyl acetate at low concentrations increased conidial germination of *B. cinerea* in vitro, but at higher concentrations, conidial germination was inhibited (Brown, 1922). Hexyl acetate at 1250 μl per liter had no effect on the in vitro conidial germination of *B. cinerea* (Wilson et al., 1987).

Acetate esters appear to be common in apple fruit, but little is known about their role in microbial interactions on the fruit. Yeasts are common inhabitants of apple fruit surfaces, and several are known antagonists of filamentous fungi that cause fruit decay in postharvest storage (Janisiewicz et al., 1994; Filonow et al., 1996; Filonow, 1998). Filonow et al. (1996) identified several yeasts that were effective in protecting apple wounds from gray mold caused by *B. cinerea*, whereas other yeasts, such as *Sporidiobolus salmonicolor* and *Tilletiopsis washingtonensis* were not. Vishniac et al. (1997) showed that *S. salmonicolor* and *T. washingtonensis* grew on low concentrations of ethyl, butyl, and hexyl acetates and hexyl-2-methyl-butanoate, which were found emanating from Golden Delicious apples; however, higher concentrations of these volatiles inhibited growth. Moreover, these volatile acetate esters were not utilized as food by yeasts that were effective antagonists of *B. cinerea* (Filonow et al., 1996). Except for the report of Vishniac et al. (1997), there has been no investigation of the role that volatile acetate esters may play in influencing the *B. cinerea*—yeast interaction in gray-mold biological control.

The objectives of this paper were to determine the effect of some volatile acetate esters commonly produced by apple on the germination of *B. cinerea* conidia and the ability of yeasts to suppress conidial germination when exposed to these acetate esters.

METHODS AND MATERIALS

Fungi and Their Culture. *Botrytis cinerea* F-J-4 and *Sporobolomyces roseus* FS-43-236 were obtained from W. J. Janisiewicz (USDA/ARS, Kearneysville, West Virginia). *Cryptococcus laurentii* BSR-Y22 was a gift of H. Vishniac (Department of Microbiology and Molecular Genetics, Oklahoma State University, Stillwater, Oklahoma), and *Saccharomyces cerevisiae* BY-1 was obtained from a packet of baker's yeast (Fleishman, Inc., San Francisco, California). Maintenance of *B. cinerea*, the production of conidia on potato–dextrose–agar (PDA), and the preparation of water suspensions of conidia have been reported elsewhere (Filonow et al., 1996). Density of conidia in suspensions were determined in a hemacytometer, and were 1×10^5 conidia/ml, unless stated otherwise. Yeasts were grown in glucose–yeast extract–peptone (GYP) broth for 24 hr at 22°C, harvested by centrifugation, and washed twice by resuspending the cells in sterile water and recentrifuging (Filonow et al., 1996). Yeast densities in suspensions were 1×10^7 CFU/ml unless noted otherwise.

Effect of Acetate Esters and Yeasts on Conidial Germination In Vitro. Suspensions of *B. cinerea* conidia (200 μl) were aseptically pipetted into a 10-mm-diam. × 10-mm-long glass cylinder resting on a piece of polycarbonate membrane (one-quarter sections cut from 47-mm-diam. membranes; 0.8 μm pore; Corning-Costar, Inc., Pleasanton, California). Conidia were deposited on the membranes by gentle suction. These membranes were then placed on Whatman filter paper disks (2.5 cm diam.; Whatman, Inc., Maidstone, England) that were saturated with 200 μl of a yeast suspension or water as the control. A filter paper disk bearing a membrane was pressed paper-side onto the inside of a sterile glass jar (500 cc; Kerr, Inc., Los Angeles, California). The top of the jar was fitted with a rubber septum. The jar was sealed, and 0, 4, 8, or 16 μl of ethyl acetate (Sigma Chemical Co., St. Louis, Missouri), butyl acetate (Fluka Chemika, Switzerland), or hexyl acetate (Fluka Chemika) were injected with a microsyringe. The acetate esters were >99.5% pure. There were three replicates per acetate ester per yeast or water treatment, and the jars were randomized on a bench.

After incubation for 24 hr at 22°C, the polycarbonate membranes were removed from the jars, placed on paper towels saturated with rose bengal to stain conidia and germlings, and destained on wet towels. The membranes were fixed with double-sided sticky tape to glass sides, and 400 conidia per membrane viewed at 400× magnification for germination.

Effect of Acetate Esters on Conidial Germination on Apples. Golden Delicious apples were purchased from a local grocery. Fruit weighing 110–125 g and free of obvious bruises were used in experiments. Mean fruit firmness was 42–44 N and soluble solids were 11.8–12.5%. Fruits were used on the day of purchase or stored (4°C) for one week or less before use. Fruits from cold storage were equilibrated at 22°C for 16–24 hr before use.

Polycarbonate membranes bearing conidia were prepared as described above. These membranes were kept moist on sterile wet Whatman filter paper (12 cm diam.) in plastic Petri dishes until use. Fruits were washed in running tap water prior to experiments, but were not dried. Three membranes were placed approximately equidistant from each other on the fruit surface. One fruit was placed inside a 500-cc glass jar, the jar sealed, and 2, 4, or 8 μl of ethyl, butyl, or hexyl acetate injected into the headspace. Jars with no acetate esters served as controls. There were five jars per acetate ester completely randomized on a bench at 22°C. After 24 hr, conidial germination on the membranes was assessed as described above.

Effect of Acetate Esters and Yeasts on Conidial Germination on Apples. Suspensions of conidia (2 × 10^5 conidial/ml) and yeasts (2 × 10^7 CFU/ml) were prepared, and 4 ml of a conidial suspension were mixed with 4 ml of a yeast suspension or water. Polycarbonate membranes bearing conidia alone or mixtures of yeasts and conidia were prepared as described above. These membranes were

kept moist, later applied to washed apples, and incubated in headspaces containing 2 μl of each acetate ester as previously described. There were five jars per acetate ester per conidia–yeast mixture. Conidial germination was measured after 24 hr.

Effect of Apples on Conidial Germination in Sealed Jars. A 400-μl drop of sterile water was pipetted into a plastic dish (30 mm diam. × 7 mm deep), and a polycarbonate membrane bearing conidia was placed on the drop. The plastic dish was placed inside a 3.9-liter jar containing one, two, or three apples. The jars were sealed with screw caps. There were five replicates per treatment. Jars without apples were the control. Jars were randomized on a bench and incubated at 22°C for 24 hr. Membranes were then removed and conidial germination assessed.

Effect of Butyl Acetate on B. cinerea *Infection of Apples in Jars.* Two wounds (3 mm diam. × 3 mm deep) were made on opposite sides of an apple at its equator by puncturing the tissue with a nail. Apples were incubated at 22°C for 24 hr, then the wounds were inoculated with 20 μl of a suspension of 2×10^5 conidia of *B. cinerea* per milliliter of water. Preparation of the conidial suspension is described elsewhere (Filonow, 1998). One apple plus a moist paper towel were put into each of 30 jars (3.9 liters) and the lids sealed. In 15 of the jars, butyl acetate (20 μl) was injected through a rubber septum into a small vial hanging below the septum. The jars were completely randomized on a bench. After incubation for 72 hr at 22°C, the jars were opened for 30 min, the paper towel exchanged with two freshly moistened towels, and the lids loosely screwed on. After four days at 22°C, the number of infected wounds on each apple and the lesion diameters were recorded.

Detection of Acetate Esters in Headspace of Jars Containing Golden Delicious Apples. Five sealed jars (3.9 liters), each containing one apple, were incubated at 23–24°C. At 24 and 48 hr the headspaces were mixed by inserting a 10-ml syringe through a septum in the jar cap, and repeatedly filling and purging the syringe. A solid-phase microextractor (SPME, Supleco, Inc., Bellefonte, Pennsylvania) was then inserted into the septum. Volatiles were adsorbed to the polydimethylsiloxane-coated fiber for 30 min, then thermally desorbed at 275°C in the injector of a gas chromatograph fitted with a DB-23 capillary column (30 m, 0.25 mm ID; J & W, Folsom, California) (Vishniac et al., 1997). The oven was programmed at 45°C for 3 min, followed by a 10°C/min increase to 155°C, and then held at 155°C for 1 min. Compounds were detected by FID, and identification was based on comparisons of retention times for SPME-adsorbed ethyl, butyl, or hexyl acetate.

Data Analysis. All experiments were repeated once. Data from experiments assessing the effect of increasing concentrations of acetate esters in a 500-cc headspace on conidial germination (Figure 1) are presented as the means ± SE for each concentration. For all other experiments, percent germination and per-

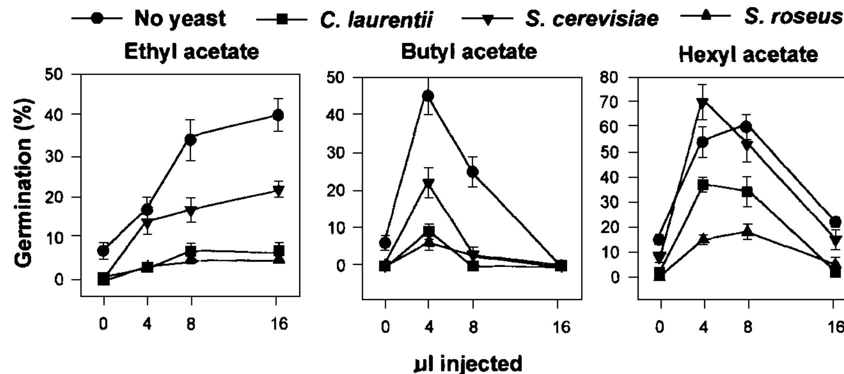

FIG. 1. Effect of ethyl, butyl, or hexyl acetate in the headspace of 500-cc jars on the germination of *B. cinerea* conidia after 24 hr at 22°C. Conidia were on polycarbonate membranes on filter paper saturated with water or yeast suspensions. Values are the means ± SE of three replicates.

cent infection data were subjected to an arcsine square-root transformation prior to analysis of variance. Means were separated using the Dunnett's test or the Student-Newman-Keul's test ($P \leq 0.05$).

RESULTS

Effect of Acetate Esters and Yeasts on Conidial Germination in Vitro. Exposure of *B. cinerea* conidia to ethyl acetate increased conidial germination from 7.0% in the no-ethyl acetate control to 40.2% at 16 µl in the headspace (Figure 1). *C. laurentii* and *S. roseus* greatly reduced this stimulatory effect, whereas *S. cerevisiae* effected an intermediate reduction.

Butyl acetate increased conidial germination to 45.4% at 4 µl in the headspace (Figure 1), but amounts greater than 8 µl reduced germination to nil. At 4 µl butyl acetate, *C. laurentii* and *S. roseus* reduced conidial germination fourfold, whereas the reduction effected by *S. cerevisiae* was twofold.

Conidia exposed to hexyl acetate exhibited maximum germination (60.2%) at 8 µl in the headspace; however, at 16 µl this stimulatory effect was nullified (Figure 1). *S. cerevisiae* did not reduce the stimulatory effect, whereas *C. laurentii* and *S. roseus* did. *S. roseus* was more effective at 4 and 8 µl hexyl acetate than was *C. laurentii.*

Effect of Acetate Esters on Conidial Germination on Apples. Acetate esters stimulated the germination of conidia on apple (Figure 2). The pattern of germination responses to individual acetate esters on apple was similar to that found

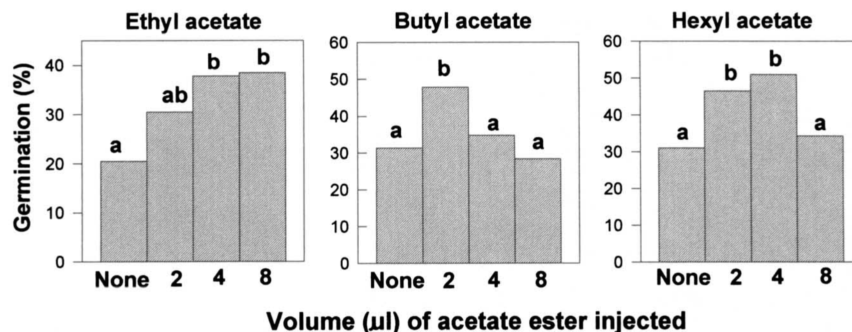

FIG. 2. Effect of ethyl, butyl, or hexyl acetate in the headspace of 500-cc jars on the germination of *B. cinerea* conidia after 24 hr at 22°C. Conidia were on polycarbonate membranes on Golden Delicious apples. Bars are the means of five replicates. Bars with the same letter are not significantly different ($P \leq 0.05$) from each other according to the Student-Newman-Keul's test.

when conidia were incubated on filter paper (Figure 1). Germination of conidia increased with increasing volumes of ethyl acetate injected into jars, but only at 4 μl was this increased germination (37.8%) significantly greater than the control (20.5%). Only 2 μl of injected butyl acetate increased germination of conidia (47.9%). Injection of 2 or 4 μl hexyl acetate increased germination of conidia, but 8 μl did not.

Effect of Acetate Esters and Yeasts on the Conidial Germination on Apple. Yeasts reduced the stimulatory effect of acetate esters on the germination of conidia on apple (Figure 3). *C. laurentii* and *S. roseus* were consistently more effective than *S. cerevisiae.*

Effect of Apples in a Sealed Jar on Germination of Conidia. Germination of conidia was increased 2.5-fold when conidia were exposed to an apple in a sealed jar (Figure 4). However, increasing the number of apples in the jar did not further increase germination of conidia.

Effect of Butyl Acetate on B. cinerea *Infection of Apples in Jars.* In both experiments, apple wounds exposed to butyl acetate had a greater incidence of infection from *B. cinerea* than did wounds that were not exposed (Table 1). The frequency of infection was increased 20–33% after exposure. Lesion diameter was not significantly increased by exposure to butyl acetate in one experiment, but it was in a second experiment.

Detection of Acetate Esters in Headspace of Jars Containing Golden Delicious Apples. Many volatile compounds emitted from apple were detected (Figure 5). Two of these, butyl acetate (BA; R_t = 4.93 min) and hexyl acetate (HA;

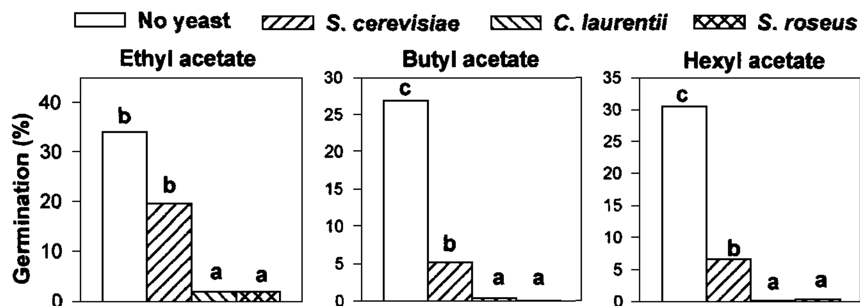

FIG. 3. Germination of *B. cinerea* conidia alone or combined with yeast cells on polycarbonate membranes on apples after 24 hr at 22°C in 500-cc jars containing 2 μl of ethyl, butyl, or hexyl acetate. Bars are the means of five replicates. Bars with the same letter are not significantly ($P \leq 0.05$) different from each other according to the Student-Newman-Keul's test.

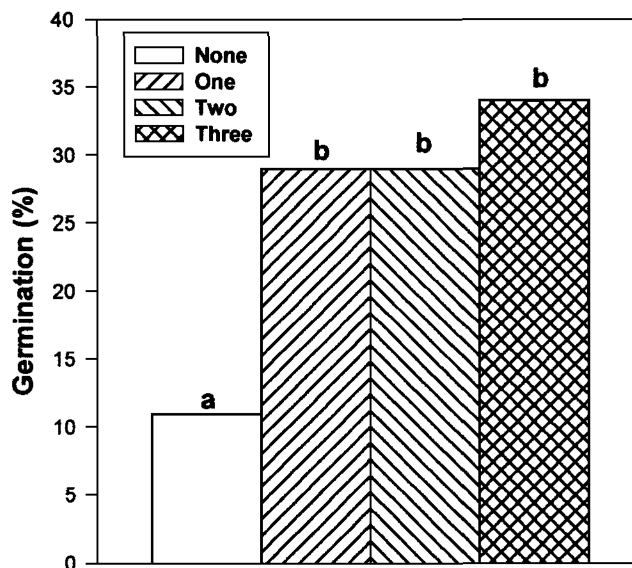

FIG. 4. Effect of number of Golden Delicious apples in a 3.9-liter jar on the germination of *B. cinerea* conidia after 24 hr at 22°C on polycarbonate membranes on water inside the jars. Bars are the means of five replicates. Bars with the same letter are not different ($P \leq 0.05$) from each other according to the Student-Newman-Keul's test.

TABLE 1. EFFECT OF EXPOSURE TO BUTYL ACETATE ON INFECTION OF GOLDEN DELICIOUS APPLES BY *Botrytis cinerea* CONIDIA[a]

	Infection frequency (%)		Lesion diameter (mm)	
	No butyl acetate	Butyl acetate	No butyl acetate	Butyl acetate
Experiment 1	50a	83b	11.7a	16.6a
Experiment 2	73a	93b	10.8a	18.5b

[a]Values are the means of 15 replicates. Means in a row followed by the same letter are not significantly different using Dunnett's Test ($P \leq 0.05$).

R_t = 8.28 min) were consistently found in the headspaces, whereas ethyl acetate (R_t = 2.62 min) was not.

DISCUSSION

Brown (1922) reported that ethyl acetate stimulated germination of *B. cinerea* conidia. I confirm this finding and newly report that butyl and hexyl acetate stimulate the germination of *B. cinerea* conidia. I found butyl and hexyl acetates, but not ethyl acetate, in the headspace of Golden Delicious apples, which agrees with the results of Vishniac et al. (1997) in their study of Golden Delicious apples from an orchard in Oklahoma. Apparently, ethyl acetate is not produced in significant quantities by Golden Delicious apples. However, Mattheis et al. (1991) found significant quantities of ethyl, butyl, and hexyl acetates and other acetate esters in the headspace of Bisbee Delicious apples.

The mechanism by which acetate esters stimulate conidial germination is not known and should be investigated. The role that acetate esters play in the decay of apples caused by *B. cinerea* was not the focus of this paper, but it most likely is complex. It is difficult to determine the fungal germination rates inside an apple wound, so I studied germination on membranes on water or on apple skin. Butyl acetate enhanced the frequency of *Botrytis* infection of apple, and this is consistent with the stimulatory effect that this ester had on conidial germination of *B. cinerea.* Greater concentrations of butyl acetate and the other esters, however, were inhibitory to conidial germination and presumably would be to *Botrytis* infection.

Apples also produce numerous other compounds that may alter the infection process. Other volatiles, such as 1-hexanol, (*E*)-2-hexenal, and 2-nonanone (Vaughan et al., 1993); some aldehydes (Hamilton-Kemp et al., 1992); and benzaldehyde (Wilson et al., 1987) are known to alter the germination of *B. cinerea* conidia. Chemicals may show synergistic effects. For instance, Eckert and Ratnayake (1994) have shown that volatile compounds emitted from

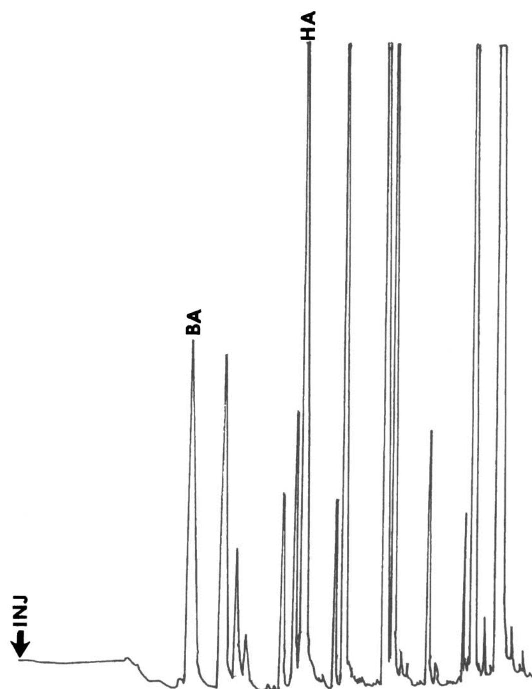

FIG. 5. Gas chromatogram of volatiles found in the headspace of 3.9-liter jars containing Golden Delicious apples. BA = butyl acetate, HA = hexyl acetate.

wounded oranges which were individually nonstimulatory to the germination of *Penicillium digitatum* conidia, stimulated germination when combined in a mixture.

Botrytis cinerea is primarily an opportunistic fungus that colonizes wounds of apples and rarely infects through intact apple skin. In wounds, sugars are readily available as nutrients for the germination of conidia (Filonow, 1998). Wounding and the infection process would presumably elicit phytoalexins, although these antifungal compounds have yet to be reported in apple. The net effect of all of these compounds on the germination of *B. cinerea* conidia makes it difficult to assess the relative contribution of a specific chemical to *Botrytis* infection in vivo. Nonetheless, it is important to understand the role that volatile chemicals in apple wounds play in pathogenesis by *B. cinerea* and other decay-producing fungi of apple fruit.

I also report for the first time that some yeasts are capable of reducing the stimulatory effect of these acetate esters on the germination of *B. cinerea* conidia, but it was beyond the scope of this study to determine the mechanism. However,

depletion by the yeasts of endogenous nutrients in *B. cinerea* conidia probably could not have played a role. The isolate (F-J-4) of *B. cinerea* used in this study is dependent on nutrients for germination, i.e., its conidia have low endogenous carbon reserves for germination (Filonow et al., 1996; Filonow, 1998). Such conidia exhibit low germination in sterile distilled water unless nutrients (or some non-nutritive stimulant) are added to the water, and they typically conserve nutrients rather than leak them to nourish other microbes (Lockwood and Filonow, 1981; Lockwood, 1986). In the present study, F-J-4 conidia germinated ca. 7–15% on sterile distilled water compared to ca. 40–60% when the conidia on water were exposed to acetate esters. Since there is a paucity of nutrients in the conidia and in distilled water, which the conidial germination rate on water showed, it is the acetate esters in the water that stimulated germination. Moreover, when not exposed to acetate esters, conidial germination on the surface of apples was ca. 20–30% compared to that on water (ca. 7–15%). If one presumes that volatiles had no effect on germination, then only nutrients on apple surfaces contributed to this two- or threefold increase in germination. Two yeasts, however, reduced germination on apple by 10-fold or greater when the apples were exposed to the acetate esters. This reduction must be due to the suppression of an effect other than a depletion or prior nutrients, or else the germination reduction would have been only two- or threefold.

Cryptococcus laurentii BSR-Y22 and *S. roseus* FS-43-238 were more effective in the biocontrol of gray mold of apple than *S. cerevisiae* BY-1 (Filonow, 1998). The yeasts differed little in their ability to compete for sugars, yet *C. laurentii* BSR-Y22 and *S. roseus* FS-43-238 reduced conidial germination more than *S. cerevisiae* BY-1, suggesting that additional factors may play a role in the biocontrol efficacy of these yeasts. The findings presented here showed that *C. laurentii* BSR-Y22 and *S. roseus* FS-43-238 were more effective than *S. cerevisiae* BY-1 in reducing the stimulatory effect of acetate esters on germination. Although it is reasonable to assume that the yeasts would exhibit the same effect in apple wounds, it may be difficult to show this experimentally.

In summary, yeasts were shown to reduce the stimulatory effect that some acetate esters have on the germination of *B. cinerea* conidia. The interactions of acetate esters with other yeasts and other decay-producing fungi of apple should be explored. In vivo methods for quantifying these interactions should be developed. Factors affecting the dynamics of acetate ester formation in apple tissue, their release as volatiles, their partitioning and diffusion in water films surrounding microbes, and their metabolic effect on microbes need attention. There are many obstacles to overcome in our understanding of the chemical ecology of apple wounds and the microbial interactions that occur within them.

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EFFECTS OF REPEATED EXPOSURE TO FOX ODOR ON LOCOMOTOR ACTIVITY LEVELS AND SPATIAL MOVEMENT PATTERNS IN BREEDING MALE AND FEMALE MEADOW VOLES (*Microtus pennsylvanicus*)

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Abstract—Following five days of baseline activity recording, voles were exposed to fox odor for 3 min each day for five days. Immediately following each daily exposure, locomotor activity levels and spatial movement patterns were assessed using an automated activity monitoring system (Digiscan system). Males displayed a significant reduction in levels of various measures of locomotor activity following exposure to fox odor on each exposure day relative to baseline levels. Males preferred the corner of the testing box significantly more on the second day of fox odor exposure relative to baseline. Although females showed only a brief reduction in the number of movements made on the first day of odor exposure, this response lasted significantly longer on each of the subsequent odor exposure days. The reliability of the reductions in activity levels displayed across days by breeding male voles supports the hypothesis that this response is adaptive. Furthermore, the results suggest that, although female voles do not generally display this behavioral response, it can be elicited in females when the predation threat is repeated in consistent context.

Key Words—Locomotor activity, meadow vole, sex difference, predator odor.

INTRODUCTION

Predation represents the leading cause of death for many small rodents and as such has led to a variety of predator detection mechanisms and antipredatory

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responses (Lima, 1998). Several species have evolved the ability to detect a predator by its odor, thus reducing the chances of actual encounters with the predator. Past work has demonstrated, both in the laboratory and in the field, that rodents display characteristic behavioral responses following exposure to a predator or its odor. In particular, predators are able to influence the levels and patterns of prey motor activity (Lima, 1998). Results of field studies have shown that small mammals avoid vegetation and traps tainted with the odors of predators, including the red fox (*Vulpes vulpes*) and weasel (*Mustela* spp.) (Calder and Gorman, 1991; Dickman and Doncaster, 1984; Sullivan et al., 1988). Similarly, increases in defensive behaviors and decreases in nondefensive behaviors have been noted in laboratory rats following exposure to predatory cat odor (Blanchard et al., 1990, 1995a,b). The results of these laboratory and field investigations have further suggested that these predator odor responses are sexually dimorphic, with males apparently displaying enhanced behavioral responses relative to females (Jedrzejewski and Jedrzejewski, 1990).

Our recent work has focused on behavioral responses of meadow voles (*Microtus pennsylvanicus*) to predator odor in the laboratory. We have demonstrated that male meadow voles display a significant reduction in locomotor activity levels following exposure to fox odor (trimethyl thiazoline; main constituent of fox anal gland secretion) but not following exposure to other aversive, pungent control odors (Perrot-Sinal et al., 1996). We have established further that this response is selective, as it is observed only in males that are reproductively active and not in either breeding female or in nonbreeding male or female meadow voles (Perrot-Sinal, 1998; Perrot-Sinal et al., 1996). It was hypothesized that this particular response is adaptive for breeding males since: (1) foxes hunt using sound cues primarily (Jedrzejewski et al., 1993) and reductions in locomotor activity levels would serve to reduce sound, and (2) breeding males may have a greater chance of encountering a predator in the wild because they have increased home-range size, higher activity levels, and likely spend more time in open areas such as runways (Madison, 1985; Madison and McShea, 1987). It may be hypothesized further that this particular behavioral response is not an essential part of the repertoire of defensive behaviors of breeding females. This may be because females either have lower activity levels and smaller home ranges, do not enter open areas as readily as males (where this defense would be most effective), and/or have evolved other behavioral responses that were not measured.

It is quite likely that in the wild rodents are repeatedly exposed to predator odor, especially when population levels of predators are high. Most past work has investigated behavioral responses following acute exposure to a predator odor. However, a few studies have examined behavioral responses in laboratory rats to more chronic predation threats. For example, male rats continued to avoid (File et al., 1993) and display a reduced amount of contact time with (Zan-

grossi and File, 1992) a cloth that had been tainted with cat odor, even after five consecutive exposures. Similarly, a detailed study examining more ethologically relevant behaviors demonstrated that increases in the use of tunnels and burrows by male laboratory rats following exposure to predation threat does not diminish after five consecutive exposures (Blanchard et al., 1990) and behavioral habituation is minimal after 20 days of 60-min exposure to a cat (Blanchard et al., 1998).

The majority of field investigations of repeated/continuous exposure to predator odors have been conducted with microtine rodents such as the meadow vole. In contrast, laboratory investigations of predator responses have been conducted primarily with laboratory rats and mice (Blanchard et al., 1990; 1995b; File et al., 1993). Here we propose to add to our understanding of defensive behaviors following predation threat by examining repeated exposure in the laboratory setting in a species other than the laboratory rat and by providing evidence for the adaptiveness of these behaviors. In the present study, we examined the effects of repeated daily exposure to fox odor on locomotor activity levels and patterns of space use in male and female meadow voles housed in mixed-sex pairs under a reproductively stimulatory photoperiod (simulating the breeding season). A detailed multivariate analysis of locomotor activity levels and patterns of space use was obtained using an automated activity monitoring system (Ossenkopp and Kavaliers, 1996). It was hypothesized that, if reductions in activity levels are adaptive for breeding males, this behavioral response would not habituate with repeated exposure. Furthermore, although breeding females do not generally display reductions in activity levels following exposure to fox odor, it was hypothesized that this behavioral response may be elicited under repeated predation threat in a consistent environment.

METHODS AND MATERIALS

Subjects

Eight male and eight female, adult, laboratory bred (fifth–sixth generation) meadow voles (40–80 g; approximately 12–15 months of age) were housed in mixed-sex pairs throughout the experiment. The animals were kept in polyethylene cages with hardwood (Beta chip) bedding in a colony room maintained at 21 ± 1°C under a long day (i.e., reproductively stimulatory) photoperiod (16L: 8D dark cycle with lights on at 06:00 hr). Since the housing conditions used were meant to simulate the breeding season (Adams et al., 1980; Lee et al., 1970; Seabloom, 1985), voles will be labeled as breeding. Information about reproductive state was gained through examining testes position in males and sex steroid levels in both sexes. Food (Agway Rat Chow and alfalfa pellets)

and water were available ad libitum. Lab chow was supplemented with carrot once a week.

Experimental Apparatus

The automated Digiscan activity monitoring system consisting of six Digiscan Animal Activity Monitors (Omnitech model RXY2CM-16, Omnitech Electronics Inc., Columbus, Ohio) was used. Activity test chambers consisted of clear Plexiglas boxes (40 × 40 × 30 cm) that had a grid of infrared beams mounted horizontally every 2.54 cm along the perimeter (16 infrared beams along each side) and 4.5 cm above the floor. The six monitors were connected to a Digiscan Analyzer (Omnitech model DCM-8) that transmitted the activity data to a microcomputer. A number of activity variables, either calculated directly by the Digiscan Analyzer or derived from the measured variables, were examined in order to provide a complete assessment across days of testing (Table 1). The validity and reliability of these measures has been previously verified (Ossenkopp et al., 1987; Sanberg et al., 1985).

TABLE 1. DESCRIPTIONS OF ACTIVITY VARIABLES MEASURED DURING BASELINE ACTIVITY MONITORING AND FOLLOWING FOX ODOR EXPOSURE IN MALE AND FEMALE MEADOW VOLES

Activity variable	Brief description
Total distance (cm)	The horizontal distance traveled by an animal during a given time sample (dependent on animal's path)
Movement time (sec)	The amount of time spent engaged in movement during a given time sample
Number of movements	The number of separate movements made by an animal during a given time sample
Center time (sec)	The amount of time spent in the center of the testing box (animal's center of gravity more than 6.35 cm away from any wall) during a given time sample
Margin time (sec)	The amount of time spent within 6.35 cm of a wall during a given time sample
Center distance (cm)	Distance traveled in the area defined as the center of the testing box (see center time)
Margin distance (cm)	Distance traveled outside of the area defined as the center of the testing box (see margin time)
Time in corners (sec)	The amount of time spent in close proximity to 2 adjoining walls of the testing box

Voles were exposed individually to the fox odor in a varnished wooden box that was divided into six equal compartments (32 × 15 × 15 cm each). The box had a Plexiglas front and lid, which was closed during exposure.

Procedure

Baseline Activity Monitoring. At mid-photoperiod, each vole was placed individually in an activity monitor and the activity measures were accumulated in 5-min time "bins" over a 30-min session (a total of six time samples). Baseline activity was monitored in this manner on five consecutive days prior to the beginning of the repeated daily odor exposures in order to habituate the voles to the apparatus.

Activity Monitoring Following Odor Exposure. On the day immediately following the end of baseline recording, voles were exposed to fox odor (2,5-dihydro-2,4,5-trimethyl thiazoline; Phero-Tech Inc.) for five consecutive days. Since previous experiments have demonstrated that activity levels following a variety of control odors, including butyric acid (Perrot-Sinal et al., 1996) and extract of almond (Perrot-Sinal, 1998), are not different from activity levels monitored during the second day of baseline activity recording, no control odor condition was used in the present experiment. Additionally, levels of activity during baseline did not change significantly across the five days of testing for males or females. Thus, activity levels following exposure to fox odor were compared to baseline levels of activity averaged across the five baseline days. This average was not different from levels of activity measured previously during baseline (Perrot-Sinal et al., 1996) and therefore was assumed not to be different from levels of activity previously measured following exposure to control odors.

Cotton swabs saturated with the odor solution were attached with masking tape inside the Plexiglas front of each compartment of the exposure box. Once all cotton swabs were in place, animals were removed from their home cages, individually placed into the compartments, and the lid was closed. Odor exposure lasted 3 min, after which the animals were removed from the compartments, placed in their home cages, and transported to an adjacent room where the activity monitoring apparatus was located. This process required approximately 1–2 min. Activity was monitored for 30 min in the same manner as during the baseline recording (see above) and took place at mid-photoperiod. Following each odor exposure trial, the wooden box was thoroughly washed using an odorless Alconox (VWR Canlab) solution and then rinsed with a baking soda solution.

Following activity monitoring, voles were returned to their home cages. The Plexiglas activity monitors were thoroughly cleaned with an Alconox solution and rinsed with a baking soda solution between each activity recording session.

Radioimmunoassay of Sex Steroids. The reproductive state of male and female voles was assessed by assaying plasma levels of sex steroid hormones.

Following the completion of the fifth day of odor-exposure testing, voles were given sodium pentobarbital (65 mg/kg, intraperitoneally) and once animals were completely anesthetized, blood was taken by transcardial puncture. Blood samples were centrifuged at 14,000 rpm (Eppendorf, model 5402) for 10 min and the resulting plasma was stored at −50°C until the time of assay. For male and female voles, plasma samples (100 μl) were assayed in duplicate for testosterone or estradiol, respectively, using commercially available ^{125}I RIA kits (Coat-a-Count, Diagnostic Products, Los Angeles, California). For the testosterone assay, the antiserum had a cross-reactivity with 5α-dihydrotestosterone of 5%, and the sensitivity of the assay was 0.025 ng/ml as calculated from the standard curve. The intraassay coefficient of variation was measured in triplicate from low, medium, and high pools and ranged from 7% to 15%. For the estradiol assay, the sensitivity of the assay was calculated to be 10.5 pg/ml and the intra-assay coefficient of variation, as measured in triplicate from low, medium, and high pools, ranged from 3% to 18%.

Data and Statistical Analyses

To assess differences in activity levels across the five days of fox odor exposure, repeated-measures analysis of variance (ANOVA) was performed for each activity variable separately with condition [baseline (average across five days of baseline activity recording) and five exposure days] and time (six time samples) as within-subject factors. These were performed separately for male and female voles.

Post-hoc tests consisted of Tukey's HSD test for all tests. A significance level of $\alpha = 0.05$ was used for all tests.

RESULTS

Reproductive Condition and Plasma Levels of Sex Steroids. All males had scrotal testes, which is a good indicator of heightened reproductive activity in small mammals (McCravy and Rose, 1992). One male and one female were excluded from the testosterone and estradiol analyses, respectively, because the plasma samples obtained were not large enough to perform the assays. The mean level of testosterone in plasma of males was 1.37 ± 0.6 ng/ml ($N = 7$), while the average level of estradiol was 15.5 ± 1.6 pg/ml in plasma of females ($N = 6$). One female was removed from all analyses because she gave birth during the course of the experiment. Her partner was retained in the experiment but was housed alone for the odor-exposure portion of the study. The plasma testosterone level of this male did not appear to be different from the other males. Levels of estradiol in females measured here were indicative of reproductively active females and higher than levels reported for reproductively quiescent females (Perrot-Sinal,

1998). Levels of testosterone in males were lower than previously reported for breeding males from the same colony but were higher than levels reported for reproductively quiescent male voles (Perrot-Sinal, 1998) and comparable to levels for reproductively active wild-caught voles (Perrot-Sinal et al., 1998). The discrepancy in testosterone levels in laboratory-bred breeding males may be due to the length of time that males were housed with the same female (two weeks in present study and 72 hr in past study). Alternatively, levels of testosterone reported here may have been suppressed by the repeated exposure to predator odor stressor. For this reason, no attempt will be made to correlate hormone levels with behavior.

Activity Levels and Patterns of Space Use Following Odor Exposure for Males. Significant condition × time interactions were found for total difference traveled [F (25, 175) = 2.33, $P < 0.001$; Figure 1A], amount of time spent in movement [F (25, 175) = 2.04, $P = 0.004$] and the number of movements made [F (25, 175) = 2.01, $P = 0.005$; Figure 1B]. The results of post-hoc analyses are summarized in Table 2. Generally, activity levels were reduced for 15–20 min of activity recording following exposure to fox odor on exposure days 1 and 2 relative to baseline. Activity also was reduced during the initial 5 min following exposure to fox odor on exposure days 3–5 relative to baseline levels.

Significant condition × time interactions also were noted for the distance traveled in the center of the test box [F (25, 175) = 1.64, $P = 0.04$; Table 1 and Figure 2A] and the distance traveled around the perimeter of the test box [F (25, 175) = 2.61, $P < 0.001$; Figure 3A]. Results of post-hoc analyses are summarized in Table 2.

There was a trend for a main effect of condition for both center time ($P = 0.07$; Figure 2B) and margin time ($P = 0.07$; Figure 3B). A significant main effect of condition was noted for corner time [F (5, 35) = 3.41, $P = 0.01$; Figure 4] and post-hoc analyses revealed that more time was spent in the four corners of the test box on day 2 of exposure relative to baseline, day 1 and day 3 of exposure. Figure 5 summarizes the time spent in the different areas of the testing box for baseline and each day of odor exposure for male voles.

Activity Levels and Patterns of Space Use Following Odor Exposure for Females. A significant condition × time interaction was found for the number of movements made [F (25, 150) = 1.6, $P = 0.04$; see Figure 6A). Results of the post-hoc analyses are summarized in Table 3. Generally, reductions in the number of movements made were most pronounced on the fifth day of odor exposure relative to baseline. There were no significant differences in levels of the other activity variables measured following exposure to the fox odor relative to baseline for females (see Figure 6B for an example of total distance traveled). Female voles did not alter their patterns of space use significantly within the testing boxes following exposure to fox odor on any exposure day relative to baseline.

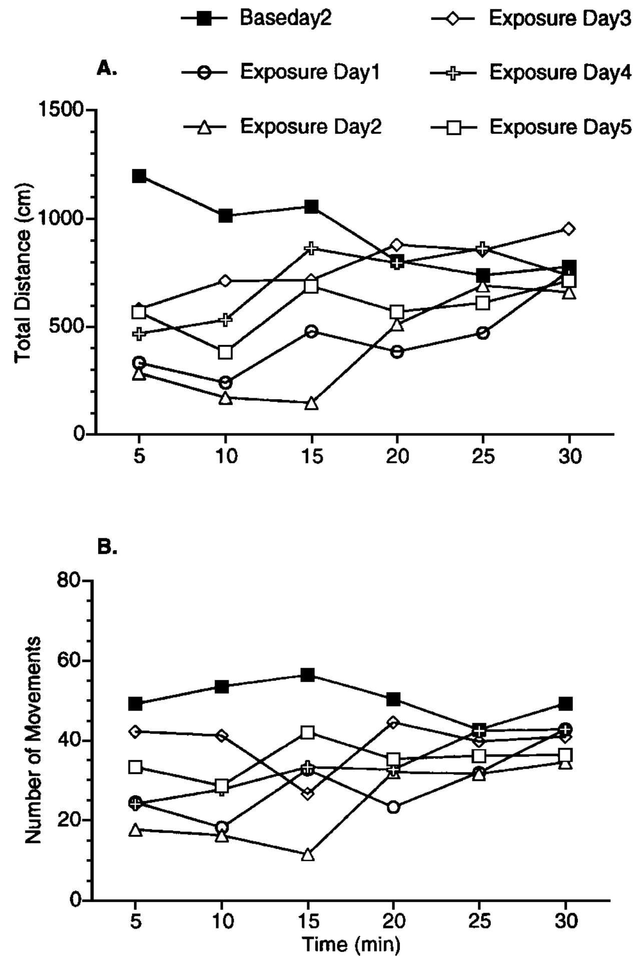

FIG. 1. (A) Total distance traveled and (B) number of movements made over 5-min intervals following exposure to fox odor on five consecutive days relative baseline in male meadow voles (N = 8). For ease of presentation, lines representing standard error of the mean have been omitted. Significant differences are summarized in Table 2.

DISCUSSION

The results of this study showed that repeated daily exposure to predator odor significantly affected the locomotor activity levels and patterns of space use by reproductive meadow voles in a sexually dimorphic manner. The major findings can be summarized as follows: (1) Breeding male meadow voles displayed

TABLE 2. SUMMARY OF ALL SIGNIFICANT REDUCTIONS IN LEVELS OF ACTIVITY AT SPECIFIC TIME POINTS FOR EACH DAY OF ODOR EXPOSURE RELATIVE TO BASELINE LEVELS FOR MALE MEADOW VOLES ($N = 8$)[a]

	Exposure day				
Activity variable	One	Two	Three	Four	Five
	5 min	5 min	5 min	5 min	5 min
Total distance (cm)	10 min	10 min		10 min[b]	10 min
	15 min	15 min			
	5 min	5 min	5 min[b]	5 min	5 min
Movement time (sec)	10 min	10 min			10 min[b]
	15 min[b]	15 min			
	5 min	5 min		5 min	
Number of movements	10 min	10 min		10 min	10 min
	15 min[b]	15 min	15 min	15 min[b]	
	20 min				
	5 min	5 min			5 min[b]
Center distance (cm)	10 min	10 min			10 min[b]
		15 min			
	5 min	5 min	5 min	5 min	5 min
Margin distance (cm)	10 min	10 min		10 min	10 min
	15 min	15 min			

[a]All significant differences are relative to baseline for same time point at $P < 0.05$ significance level.
[b]$P < 0.01$.

a significant reduction in levels of various measures of locomotor activity following exposure to fox odor relative to baseline on each of five consecutive odor exposure days. (2) The magnitude of the reduction in activity levels displayed by males changed with repeated exposure such that it was most pronounced on the first and second days of exposure, was somewhat attenuated on the third day, and then became more pronounced again on the fourth and fifth days. (3) The amount of time spent in the corners of the testing box (i.e., the "safest" places in the box) was significantly greater on the second day of exposure relative to baseline in males. (4) Although breeding female meadow voles displayed only a brief (5 min) reduction in the number of movements made on the first day of odor exposure, this reduction lasted significantly longer on each of the subsequent odor exposure days.

The reductions in activity levels following exposure to fox odor relative to baseline levels in male rodents are consistent with the results of past laboratory studies (Perrot-Sinal et al., 1996; Vernet-Maury et al., 1984). Previously, we demonstrated that levels of locomotor activity were reduced in male meadow voles following exposure to fox odor relative to various control odors (Perrot-Sinal et al., 1996), but only when males were in a reproductively active

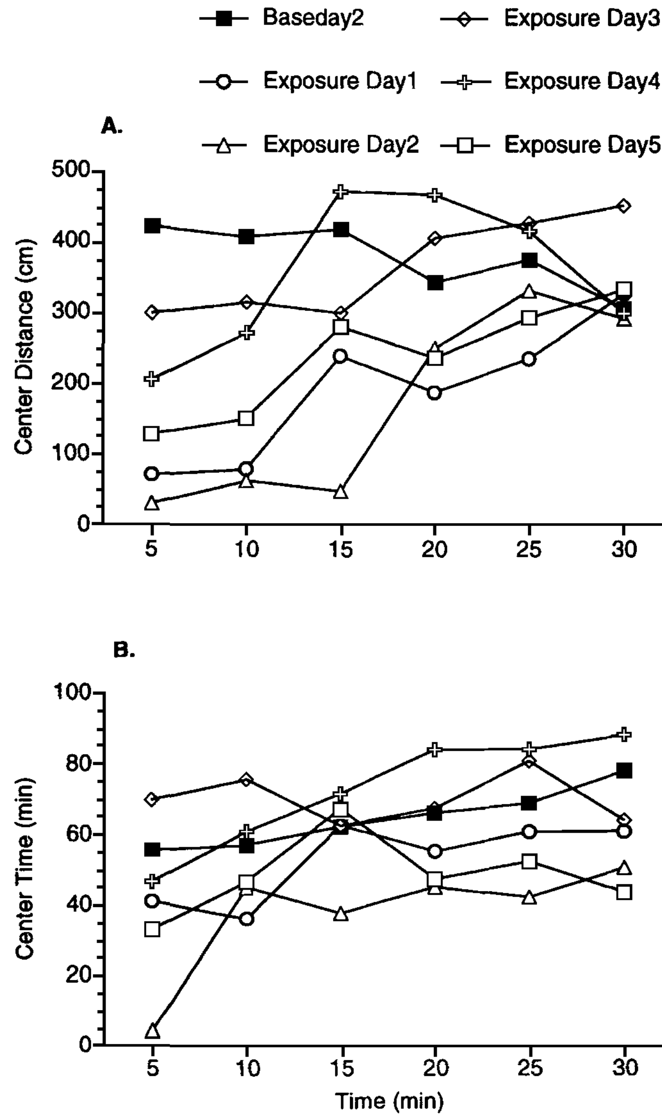

FIG. 2. (A) Distance traveled and (B) amount of time spent in the center of the testing boxes over 5-min intervals following exposure to fox odor on five consecutive days relative baseline in male meadow voles (N = 8). For ease of presentation, lines representing standard error of the mean have been omitted. Significant differences are summarized in Table 2.

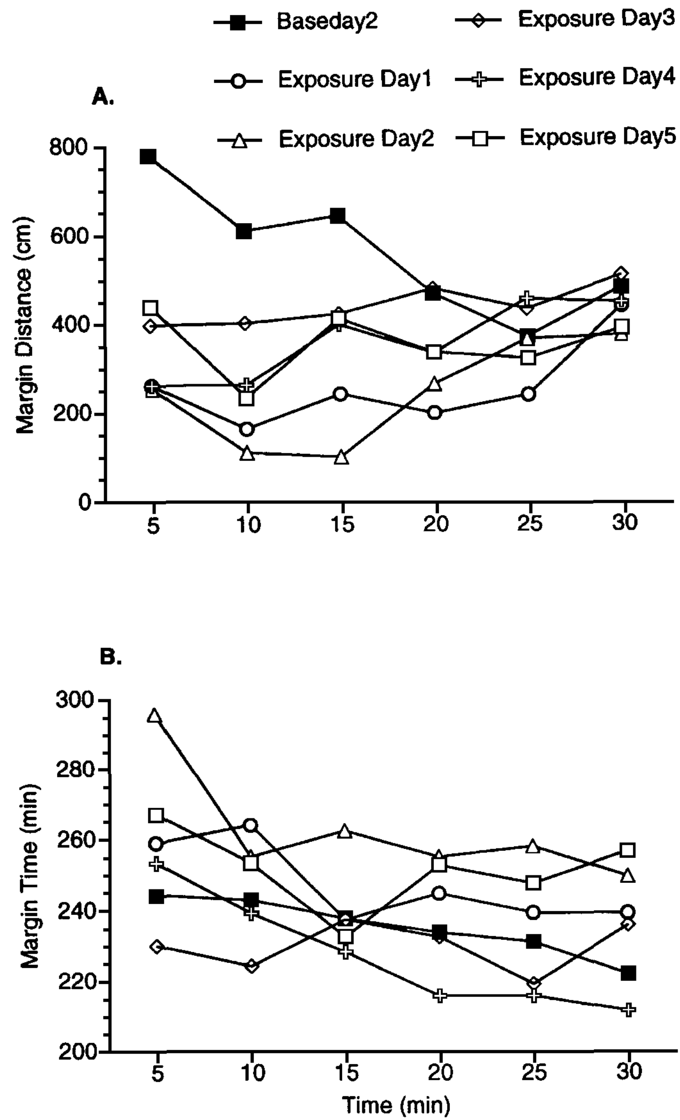

FIG. 3. (A) Distance traveled and (B) amount of time spent along the perimeter of the testing boxes over 5-min intervals following exposure to fox odor on five consecutive days relative baseline in male meadow voles (N = 8). For ease of presentation, lines representing standard error of the mean have been omitted. Significant differences are summarized in Table 2.

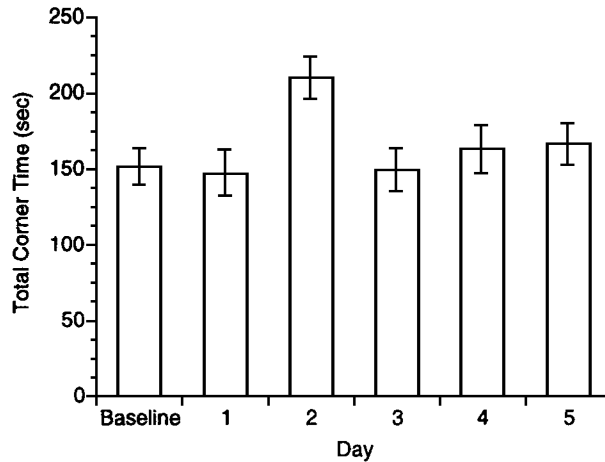

FIG. 4. Amount of time spent in the corners of the testing boxes over 5-min intervals following exposure to fox odor on five consecutive days relative baseline in male meadow voles (N = 8).

state (breeding) (Perrot-Sinal, 1998). Males housed under a reproductively nonstimulatory short photoperiod (nonbreeding state) did not display a reduction in activity levels following acute exposure to fox odor. We hypothesized that this response might be adaptive selectively for breeding males since they are more active and, in the wild, have expanded home ranges relative to nonbreeding males (Madison, 1985; Madison and McShea, 1987). It has been demonstrated that increased mobility is associated with increased predation in free-ranging voles (Norrdahl and Korpimaki, 1998).

Although we have not tested responses of wild voles using this paradigm, we believe that responses of wild voles would be similar to those of our laboratory voles for two main reasons. First, laboratory voles tested in this study and other similar investigations from our laboratory (Perrot-Sinal, 1998; Perrot-Sinal et al., 1996) were only a few generations removed from a wild population. Second, we have measured basal levels of locomotor activity in wild male voles and found them to be similar to what we find in laboratory voles in terms of absolute levels and relation to plasma levels of testosterone (Perrot-Sinal et al., 1998).

The present results expand upon our past work by demonstrating that reductions in male activity levels following fox odor exposure do not habituate with repeated fox odor exposures. This finding is important because it lends support for the hypothesis that this response is adaptive for breeding males in this context. Thus, in an open area that provides no opportunity for shelter (such as the

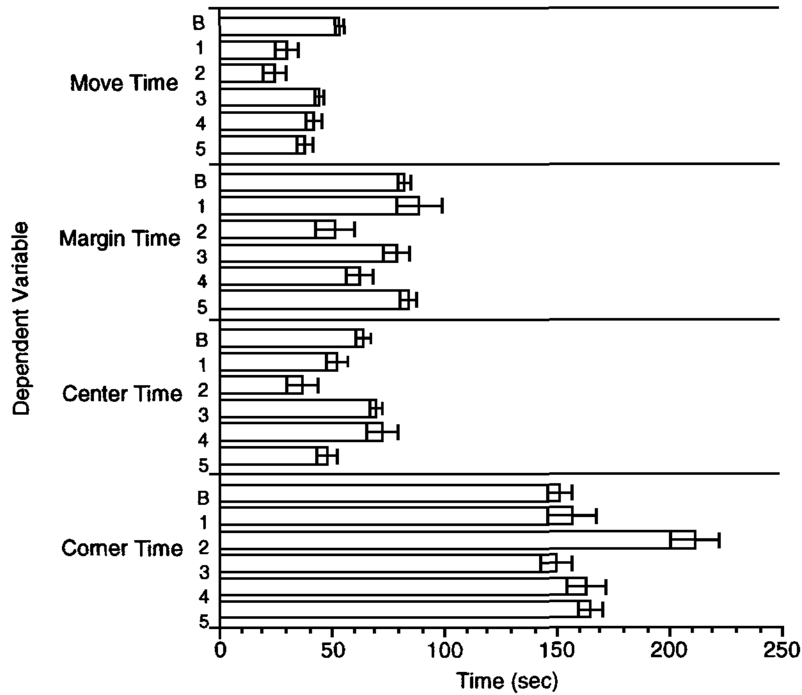

FIG. 5. Activity profile comparing various activity measures collapsed across 30 min of testing for days 1–5 of fox odor exposure relative to baseline (B) recording in male meadow voles (N = 8). Horizontal lines represent standard error of the mean (SEM).

TABLE 3. SUMMARY OF SIGNIFICANT REDUCTIONS IN LEVELS OF ACTIVITY AT SPECIFIC TIME POINTS FOR EACH DAY OF ODOUR EXPOSURE RELATIVE TO BASELINE LEVELS FOR FEMALE MEADOW VOLES (N = 7)[a]

	Exposure day				
Activity variable	One	Two	Three	Four	Five
Number of movements	5 min	5 min 10 min 20 min[b]	5 min 10 min	5 min 10 min 20 min	5 min 10 min[b] 20 min 25 min[b]

[a]All significant differences are relative to baseline for same time point at $P < 0.05$ significance level.
[b]$P < 0.01$.

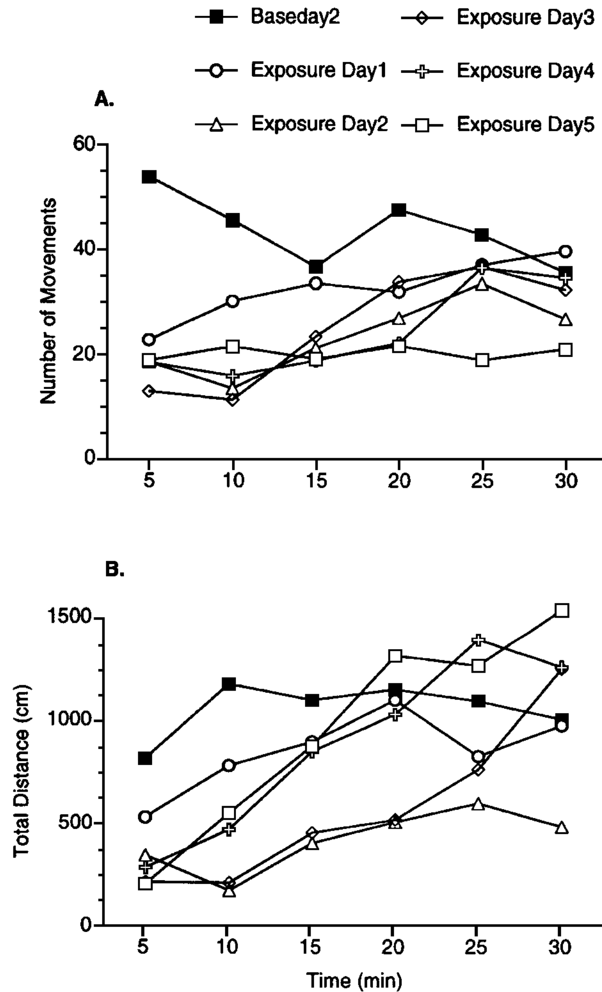

FIG. 6. (A) Number of movements made and (B) total distance traveled over 5-min intervals following exposure to fox odor on five consecutive days relative baseline in female meadow voles ($N = 7$). For ease of presentation, lines representing standard error of the mean have been omitted. Significant differences are summarized in Table 3.

testing box), this particular behavioral response was observed consistently in breeding male meadow voles. A behavioral response elicited by predator odor that results effectively in the avoidance of encounters with the predator would, no doubt, be selected for in wild populations of rodents and other species of animals (Lima, 1998). Interestingly, the magnitude of the response observed in

the present study changed somewhat over time such that on the third day of exposure, the reduction in activity levels was not as pronounced. However, on the fourth and fifth days of exposure, reductions in activity levels were as pronounced as the initial two days for most activity variables. These results are consistent with those of prior investigations examining the responses of laboratory rats to a cat or cat odor. Exposure to cat odor elicited shelter use after five days of daily exposure (File et al., 1993). Increases in the use of tunnels and burrows by male laboratory rats following exposure to predation threat does not diminish after five consecutive exposures, although there are qualitative changes across days (Blanchard et al., 1990). Our results also agree with the suppression of nondefensive behaviors evident even after 20 days of daily exposure to a cat (Blanchard et al., 1998).

We have shown previously that levels of activity following exposure to various control odors were not different from levels measured during baseline recording (Perrot-Sinal, 1998; Perrot-Sinal et al., 1996). These results suggest that the reductions in activity levels are specific to the predator odor stimulus. This specificity is consistent with past work on the electrophysiology of the dentate gyrus of the hippocampus, which has olfactory connections (Heale et al., 1994). The study of Heale et al. (1994) demonstrated that fast wave bursts could be elicited from the dentate gyrus of male rats selectively in response to odors of fox and weasel but not following exposure to other aversive, pungent odors. Thus, there exist specific physiological/behavioral systems that have evolved to respond selectively to the odor of predators.

In addition to examining levels of locomotor activity in the present study, spatial movement patterns within the test boxes were monitored in an attempt to determine whether voles display an increased thigmotaxic response following exposure to fox odor. Thigmotaxis can be described as "wall-hugging" behavior and has been suggested to be a part of an animals natural repertoire of defensive behaviours (Treit and Fundytus, 1989). In an open area, this behavior may be adaptive since a wall may provide some means of protection. In the present study, there was a trend for male voles to spend less time in the center of the test box and more time along the margin of the box following exposure to fox odor relative to baseline, at least on exposure days 1 and 2. Additionally, it was found that males spent significantly more time in the corners of the test box on the second day of exposure relative to baseline. Thus, the second exposure to the fox odor appeared to elicit the most fear- or anxiety-related behaviors in males, which was manifested in their spending increased amounts of time in the "safe" place in the testing box (i.e., beside two walls).

Similar to our past findings, female meadow voles in the present study did not display a significant reduction in activity levels for most activity variables measured (Perrot-Sinal, 1998; Perrot-Sinal et al., 1996). However, females in the present study did display a significant reduction in the number of move-

ments made during the initial 5 min of the test hour following exposure to fox odor on the first day of odor exposure relative to baseline. This transient reduction is somewhat inconsistent with our past work, although we previously noted a trend for breeding females to display a reduction in this measure previously (Perrot-Sinal, 1998). These male–female differences in response to predator odor are consistent with sex differences in acute predator responses in rats and mice (Blanchard et al., 1991, 1995b).

Interestingly, the degree of reduction in number of movements became more pronounced with repeated exposure to the fox odor, suggesting that the females were able to adapt their behavioral response. Thus, although female voles did not display a reduction in activity levels following acute fox odor exposure, as male voles do, they were able to display this behavioral response, at least to some extent, when predation threat was repeated in a stable context. Females did not show any changes in spatial movement patterns following exposure to fox odor on any of the exposure days relative to baseline. In both the breeding and nonbreeding season, females inhabit smaller home ranges and display reduced levels of locomotor activity relative to breeding males, thus reducing their likelihood of encounter with a fox. Future studies that examine the use of shelter within an open field following predation threat may illuminate female responses in an acute exposure situation.

In conclusion, various measures of locomotor activity levels were reduced significantly following exposure to fox odor on each of five consecutive exposure days in breeding males. This lends support to the hypothesis that this behavioral response is adaptive for breeding male voles. While female meadow voles displayed a transient reduction in the number of movements made following exposure to fox odor on the first exposure day, this response became more pronounced across the five exposure days. This increase in reduced activity suggests that, when faced with this threat in a consistent context, breeding females are able to alter their behavioral response, to some extent, to be more effective in that context. Taken together, these findings provide important information about defensive behaviors in the face of repeated predation threat in the meadow vole. Future work in this area should focus on examining this behavioral response following exposure to live predators and in varying contexts (i.e., access to shelter).

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VOLATILE STIMULI RELATED TO FEEDING ACTIVITY OF NONPREY CATERPILLARS, *Spodoptera exigua*, AFFECT OLFACTORY RESPONSE OF THE PREDATORY MITE *Phytoseiulus persimilis*

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Abstract—The effect of volatiles related to feeding activity of nonprey caterpillars, *Spodoptera exigua*, on the olfactory response of the predatory mites *Phytoseiulus persimilis* was examined in a Y-tube olfactometer. At a low caterpillar density (20 caterpillars on 10 Lima bean leaves), the predators were significantly more attracted to volatiles from infested leaves on which the caterpillars and their products were present or from infested leaves from which the caterpillars and their products had been removed when compared to volatiles from uninfested leaves. The predators, however, significantly avoided odors from 20 caterpillars and their products (mainly feces) removed from bean leaves. In contrast, at a higher caterpillar density (100 caterpillars on 10 Lima bean leaves), the predators avoided volatiles from caterpillar-infested bean leaves. Volatiles from infested leaves from which the caterpillars and their products had been removed were not preferred over volatiles from uninfested leaves. Volatiles from feces collected from 100 caterpillars were strongly avoided by the predators, while the behavior of the predatory mites was not affected by volatiles from 100 caterpillars removed from a plant. The data show that carnivorous arthropods may avoid nonprofitable herbivores. This avoidance seems to result from an interference of volatiles from herbivore products with the attraction to herbivore-induced plant volatiles.

Key Words—Tritrophic interactions, Lima bean, *Phytoseiulus persimilis*, *Tetranychus urticae*, *Spodoptera exigua*, Acari, Lepidoptera, infochemicals, herbivore-induced plant volatiles, nonprey herbivores, feces.

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INTRODUCTION

Plant–herbivore complexes emit volatile chemicals that may orginate from the herbivores, their products (such as feces), the plants, and/or interactions between plant and herbivores. These volatiles may be utilized as infochemicals by carnivorous enemies of the herbivores during searching for food. The significance of herbivore-induced plant volatiles in prey location and/or prey selection by predatory mites or parasitoid wasps is well-established (for reviews see Dicke, 1994; Dicke et al., 1998; Takabayashi and Dicke, 1996; Turlings et al., 1993a, 1995). In addition, odors from herbivore feces can be used by predatory mites (Sabelis et al., 1984) or by parasitoids (Turlings et al., 1991a; Steinberg et al., 1993; Geervliet et al., 1994; Agelopoulos and Keller, 1994; Agelopoulos et al., 1995; Röse et al., 1997) during prey location. However, herbivore feces elicit much weaker responses than herbivore-induced plant volatiles (Sabelis et al., 1984; Turlings et al., 1991a; Steinberg et al., 1993; Geervliet et al., 1994; Agelopoulos and Keller, 1994).

Although most studies on the response of carnivorous arthropods to herbivore-infested plants relate to attraction to plants infested by a suitable prey or host, some studies have also addressed the response to plants infested by herbivores that cannot be exploited by the carnivores (Dicke and Groeneveld, 1986; Turlings et al., 1993b; Geervliet et al., 1996; Powell et al., 1998). These studies recorded either an attraction (Turlings et al., 1993b; Geervliet et al., 1996) or no effect (Dicke and Groeneveld, 1986; Powell et al., 1998) of volatiles from plants infested by unsuitable herbivores. For instance, the parasitoid *Aphidius ervi* is attracted to faba bean plants infested by its host, the aphid *Acyrthosiphon pisum*, but not to faba bean plants infested by the nonhost aphid *Aphis fabae* (Powell et al., 1998). Plants that are infested by one of different herbivores can emit volatile blends that are specific for the herbivore that infests the plant (Takabayashi et al., 1991, 1995; Geervliet et al., 1997; Powell et al., 1998; De Moraes et al., 1998; Dicke, 1999). It is adaptive for carnivores to exploit these differences, especially when they relate to herbivores that largely differ in profitability.

Avoidance of herbivore-infested plants by carnivores has never been recorded. Yet, avoidance of plants that harbor competitors has recently been reported for carnivorous arthropods (Janssen et al., 1997). It may be adaptive to avoid plants infested by nonprey herbivores, especially when the nonprey herbivore poses a risk to the carnivore.

We have studied the effect of volatiles from Lima bean plants infested by beet armyworm caterpillars, *Spodoptera exigua* (Lepidoptera: Noctuidae), on the behavior of the predatory mite *Phytoseiulus persimilis* (Acari: Phytoseiidae). This predatory mite is a specialist that feeds almost exclusively on spider mites in the genus *Tetranychus*, such as the two-spotted spider mite *T. urticae* (Acari: Tetranychidae). Beet armyworm caterpillars cannot serve as food to the preda-

tors. *Tetranychus urticae* and *S. exigua* are both polyphagous herbivores that may infest the same host plant. The predator is strongly attracted to *T. urticae*-infested Lima bean plants (Sabelis and Van de Baan, 1983; Dicke et al., 1990, 1993; Takabayashi et al., 1994). Volatiles produced and emitted by the plants are most important in this attraction (Dicke et al., 1990; Takabayashi et al., 1994), while volatiles from spider-mite feces are only slightly attractive (Sabelis et al., 1984). In contrast, it is unknown whether the predators respond to volatiles related to the nonprey *S. exigua* caterpillars. *Spodoptera exigua* caterpillars are well known to induce plant volatiles that attract the parasitoid *Cotesia marginiventris*. This has been established for a range of plant species, but Lima bean plants have never been used (Turlings et al., 1990, 1991a, 1993a).

In this study, we investigated the olfactory response of *P. persimilis* to: (1) volatiles from Lima bean plants infested with *S. exigua* larvae on which the caterpillars were still present or from which the caterpillars had been removed just prior to the experiment, (2) volatiles from the caterpillars, and (3) volatiles from caterpillar products such as feces and silk. We demonstrate that volatile infochemicals from plants attract the predators while volatiles from caterpillar feces are avoided.

METHODS AND MATERIALS

Plants

Lima bean plants (*Phaseolus lunatus* cv. Sieva) were grown in sterilized soil in a greenhouse (20–30°C, 50–70% relative humidity, and a photoperiod of at least 16 hr). Young primary leaves from 12- to 18-day-old plants were used in the experiments.

Predators

The predatory mites *Phytoseiulus persimilis* were mass-reared on bean leaves infested with the two-spotted spider mites, *Tetranychus urticae*, in a greenhouse (20–30°C, 50–70% relative humidity, and a photoperiod of at least 16 hr). Standardized experimental predators were obtained through the following procedure. Predator eggs of the same age were obtained by placing five fertilized females from the mass-rearing on an infested leaf in each of a number of Petri dishes (9.2 cm diameter) in a climate room (23 ± 1°C, 50–70% relative humidity, and 16L:8D). After two days, the adult females were removed, while their offspring were reared in the Petri dish on the spider mites on a bean leaf for seven or eight days. Prior to an experiment, 10–20 adult female predators (8–10 days old) were introduced into a Petri dish, containing a moist filter paper but no food or plant leaf, for 60 ± 30 min in a climate room (23 ± 1°C, 50–70% relative humidity, and 16L:8D) and then used in the olfactometer bioassay.

Nonprey Herbivores

Beet armyworms, *Spodoptera exigua*, an herbivore that cannot serve as prey to *P. persimilis*, were reared on a semiartificial diet (Smits, 1987) in a climate cabinet (27 ± 2°C, 70–80% relative humidity, and 16L:8D). Eggs were transferred to a Lima bean leaf in a Petri dish in a climate room (23 ± 1°C, 50–70% relative humidity, and 16L:8D). Most eggs hatched after three days, while larvae were collected after various time intervals to be used in the preparation of odor sources.

Odor Sources

Nine different types of odor source were used.

1. Infested Lima Bean Leaves, 2 Caterpillars Per Leaf. A freshly cut uninfested Lima bean plant with two primary leaves was put with the stem in a glass vial (1.8 cm diameter, 7 cm high) filled with water. Two first-instar larvae of *S. exigua* (within 24 hr after hatching) were confined to each leaf within a clip cage (2 cm diameter, 1.5 cm high, upper and lower surface were covered with parafilm and fine mesh, respectively) and inoculated for three days in a climate room (23 ± 1°C, 50–70% relative humidity, and 16L:8D). Every day, the last time just before using the plants in the bioassay, the position of each clip cage was changed. A caterpillar that had escaped from a clip cage was replaced by a new one of the same age. Just before the bioassay experiments, the stem of each plant was wrapped in moist cotton wool. Five infested plants (20 caterpillars and their residues, mainly feces, in 10 cages on 10 leaves in total) with clean moist cotton wool were used as odor source.

2. Infested Lima Bean Leaves, 10 Caterpillars per Leaf. Ten first-instar caterpillars were introduced per leaf (five caterpillars per clip cage, two clip cages per leaf) for three days. Five infested plants (100 caterpillars and their residues in 20 cages on 10 leaves in total) with clean moist cotton wool were used as odor source. Other conditions and procedures were the same as described above under 1.

3. Previously Infested Lima Bean Leaves, 2 Caterpillars per Leaf. Two first-instar caterpillars within 24 hr after hatching were confined to each leaf with a clip cage and inoculated for three days. Just before the use in the bioassay, the cages including the 20 caterpillars and their residues were removed. Residues on the leaves, mainly fecal pellets, were removed by using a fine paint brush and moist cotton wool. Five previously infested plants with clean moist cotton wool (no residues) wrapped around the stem were used as odor source. Other conditions and procedures were the same as described above under 1.

4. Previously Infested Leaves, 10 Caterpillars per Leaf. Ten first-instar caterpillars (within 24 hr after hatching) were confined to each leaf with two clip cages for 3 days. Just before use in the bioassay, the 100 caterpillars and

their residues in the clip cages and other residues on the leaves were removed with a fine brush and moist cotton wool. Five previously infested plants with clean moist cotton wool were used as odor source. Other conditions and procedures were the same as described above under 1.

5. Caterpillars and Their Products, 20 Larvae. Two first-instar caterpillars (within 24 hr after hatching) were confined to each leaf with a clip cage and incubated for three days. Just before the use in the bioassay, the 20 caterpillars and their residues in the clip cages were collected. Other residues on the leaf surface were also collected by using moist cotton wool. Ten clip cages, including the caterpillars and their residues, and the moist cotton wool containing residues were used as odor source. Other conditions and procedures were the same as described above under 1.

6. Caterpillars, 100 Larvae. Ten first-instar caterpillars within 24 hr after hatching were confined to each leaf with two clip cages and inoculated for three days. The 100 caterpillars, collected just before the use in the bioassay, plus clean moist cotton wool (no residues) were used as odor source. Other conditions and procedures were the same as described above under 1.

7. Residues, Produced by 100 Larvae. Ten first-instar caterpillars within 24 hr after hatching were confined to each leaf with two clip cages and inoculated for three days. Just before the use in the bioassay, the cages including the residues produced by the 100 caterpillars were collected. Other residues on the leaves were also collected by using moist cotton wool. The residues in the cages and on the moist cotton wool were used as odor source. Other conditions and procedures were the same as described above under 1.

8. Uninfested Lima Bean Leaves. Five uninfested plants with clean moist-cotton wool (no residues) around the stem were used as odor source. Other conditions and procedures were the same as described above under 1.

9. Clean Air. Clean moist cotton wool was used as odor source.

Bioassay

A closed-system Y-tube olfactometer was used. The airflow through each olfactometer arm was 4 liters/min, which was checked with a flowmeter. Details of the olfactometer set up are given by Takabayashi and Dicke (1992). The following experiments were conducted.

Experiment 1: low caterpillar density (2 caterpillars per leaf, 20 caterpillars in total).

Exp. 1a: 10 infested leaves (2 caterpillars per leaf) vs. 10 uninfested leaves.
Exp. 1b: 10 previously infested leaves (2 caterpillars per leaf) vs. 10 uninfested leaves.
Exp. 1c: 20 caterpillars and their feces vs. clean air.

Experiment 2: high caterpillar density (10 caterpillars per leaf, 100 caterpillars in total).

Exp. 2a: 10 infested leaves (10 caterpillars per leaf) vs. 10 uninfested leaves.
Exp. 2b: 10 previously infested leaves (10 caterpillars per leaf) vs. 10 uninfested leaves.
Exp. 2c: 100 caterpillars vs. clean air.
Exp. 2d: Feces produced by 100 caterpillars vs. clean air.

Predators were individually introduced at the start point on an iron wire, which was positioned in the center of the glass tube. The behavior of a predator on the wire was observed for a maximum of 5 min. The observation was terminated when the predator reached the far end of one of the arms. Predators that did not reach the end of either arm (classified as making no choice) were excluded from the statistical analysis. The connections of odor source containers (2-liter conical flasks) to the olfactometer arms were exchanged after every five mites. In every experiment, a total of more than 60 predators was tested over four days in an experimental room at 22 ± 2°C.

The data were subjected to a chi-square test. The null hypothesis was that the predators had a 50 : 50 distribution over the two odor sources.

RESULTS

Experiment 1: Low Caterpillar Density (2 Caterpillars per Leaf, 20 Caterpillars in Total). The predatory mites were significantly more attracted to volatiles from caterpillar-infested leaves than to volatiles from uninfested leaves (Figure 1). The same result was obtained for the response to previously infested leaves from which the caterpillars and their residues had been removed (Figure 1). However, the predators significantly avoided odors from 20 caterpillars plus their residues when offered versus clean air (Figure 1).

Experiment 2: High Caterpillar Density (10 Caterpillars per Leaf, 100 Caterpillars in Total). At a high caterpillar density, the predatory mites avoided the caterpillar-infested plants. They significantly preferred the odors from uninfested leaves over those from caterpillar-infested leaves (Figure 2). After removal of caterpillars and their residues from infested leaves, the predators had no preference when offered volatiles from uninfested leaves vs. those from previously infested leaves (Figure 2). Volatiles from 100 caterpillars isolated from their host plant did not evoke an avoidance response. However, the predators clearly avoided odors from feces produced by 100 caterpillars when offered vs. clean air (Figure 2).

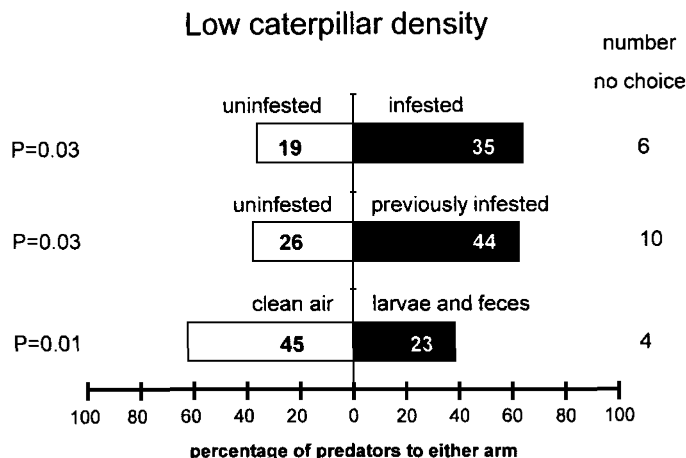

FIG. 1. Response of adult female *P. persimilis* in a Y-tube olfactometer towards volatiles from Lima bean leaves infested for three days with a low density of *S. exigua* caterpillars or to volatiles from components of this plant–herbivore complex. Infested leaves: 10 leaves with 2 caterpillars per leaf each. Larvae: 20 caterpillars removed from infested leaves. Feces: all visible residues collected from 10 leaves that had been infested with 2 caterpillars each for three days. Numbers in bar segments represent the number of predators that chose either olfactometer arm.

DISCUSSION

Our results demonstrate that volatiles related to a nonprey herbivore affect the olfactory response by the predatory mites and that herbivore numbers affected the type of behavioral response. At a low caterpillar density (20 caterpillars on 10 leaves), odors from infested or previously infested leaves attracted the predators, while volatiles from the caterpillars plus their residues repelled them. This indicates that plant volatiles induced by *S. exigua* caterpillars were able to attract the predators. At least four components of plant volatiles from Lima bean plants induced by the two-spotted spider mites *T. urticae* [(3*E*)-4,8-dimethyl-1,3,7-nonatriene, linalool, (*E*)-β-ocimene and methyl salicylate] are effective in attracting *P. persimilis* in an olfactometer (Dicke et al., 1990). The blends of volatiles emitted by plants of the same species that are infested by different herbivore species are often qualitatively similar, but differ with respect to the ratio of blend components (Takabayashi et al., 1991; DeMoraes et al., 1998). This seems to be true for Lima bean plants as well. Lima bean plants infested with two-spotted spider mites or with western flower thrips (*Frankliniella occidentalis*) emit qualitatively similar, but quantitatively dissimilar volatile blends (M.

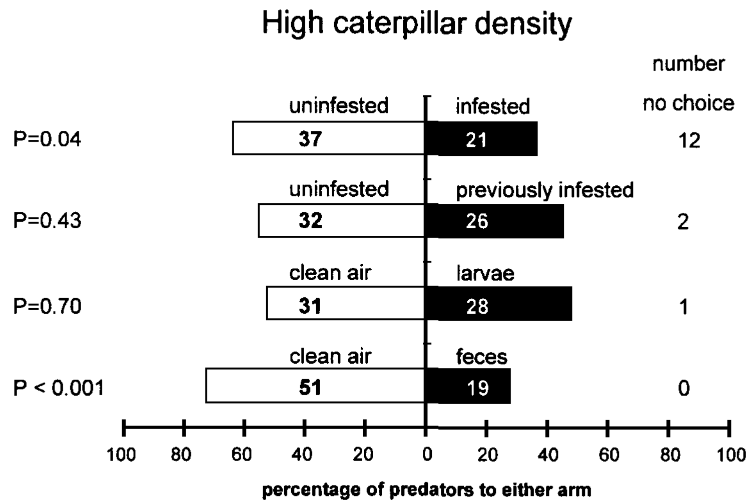

FIG. 2. Response of adult female *P. persimilis* in a Y-tube olfactometer towards volatiles from Lima bean leaves infested for three days with a high density of *S. exigua* caterpillars or to volatiles from components of this plant–herbivore complex. Infested leaves: 10 leaves with 10 caterpillars per leaf each. Larvae: 100 caterpillars removed from infested leaves. Feces: all visible residues collected from 10 leaves that had been infested with 10 caterpillars each for three days. Numbers in bar segments represent the number of predators that chose either olfactometer arm.

A. Posthumus and M. Dicke, unpublished data). It is likely that this is also true for volatiles from Lima bean plants infested with *S. exigua.*

Interestingly, volatiles from caterpillar feces are avoided by *P. persimilis.* This was true for feces from 20 as well as 100 caterpillars. Repellent effects of fecal volatiles overruled the attraction to plant volatiles at the high density of 100 caterpillars on 10 leaves but not at the low density of 20 caterpillars on 10 leaves. Thus, the relative contribution of fecal volatiles vs. plant volatiles in the total blend seems to be affected by caterpillar number. Headspace analysis of feces produced by 15 third-instar *S. exigua* caterpillars overnight while feeding on corn seedlings did not result in the detection of volatiles, while the plants damaged by the caterpillars emitted large amounts of volatiles (Turlings et al., 1991b). Although it is difficult to compare this to the amount of volatiles emitted by 20 first-instar larvae feeding for three days on bean leaves, it indicates that the amount of volatiles emitted by larval feces is much lower than that emitted by the plant tissue damaged by the same larvae. In a behavioral study on the parasitoid *Cotesia rubecula* towards volatiles from feces of *Pieris rapae* caterpillars, an increase in response was recorded with increased amounts of feces used as

odor source (Agelopoulos et al., 1995). Although odors from previously infested leaves at a high caterpillar density (10 caterpillars per leaf) did not attract the predators (Figure 2), this does not mean that plant volatiles induced by these caterpillars were not effective in attracting them. Previously infested plants still smelled of caterpillar residues to us, even though all visible residues had been carefully removed. Thus, it is likely that invisible caterpillar products were still present on the leaf surface and affected the response to herbivore-induced plant volatiles.

Our results demonstrate that volatile infochemicals from caterpillars interfere with the response of the predators to infochemicals from plants. The degree of interference is affected by caterpillar density. At a low caterpillar density, the effect of attractive plant volatiles was more important than that of repellent volatiles from caterpillar feces. In contrast, at a high caterpillar density, repellent volatiles from caterpillar feces had the most important effect on predator behavior, resulting in the avoidance of caterpillar-infested leaves.

The attraction of *P. persimilis* towards Lima bean plants infested with nonprey caterpillars at a low density suggests that the predatory mites cannot discriminate between plants infested with prey such as *T. urticae* and plants infested with nonprey. However, to know whether this is true, experiments are needed in which Lima bean plants infested with *T. urticae* are offered vs. Lima bean plants infested with *S. exigua.* Discrimination between plants infested with different herbivore species may depend on previous experiences (Turlings et al., 1993a; Geervliet et al., 1998; Vet et al., 1998; Dicke, 1999). In this study the predatory mites had experience with odors from plants infested with *T. urticae* and thus the attraction to *S. exigua*-infested Lima bean plants may be the result of generalization (cf. Smith, 1993; Vet et al., 1998). To understand why *P. persimilis* is attracted to volatiles from plants infested with a nonprey herbivore, an investigation is needed of the composition of volatiles from Lima bean plants induced by *S. exigua* caterpillars and the olfactory response of *P. persimilis* in two-choice setups with different plant–herbivore complexes as odor source and with predators that have had different types of experience.

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TRADE-OFFS IN PLANT DEFENSE AGAINST PATHOGENS AND HERBIVORES: A FIELD DEMONSTRATION OF CHEMICAL ELICITORS OF INDUCED RESISTANCE

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Abstract—Two signaling pathways, one involving salicylic acid and another involving jasmonic acid, participate in the expression of plant resistance to pathogens and insect herbivores. In this study, we report that stimulation of systemic acquired resistance in field-grown tomato plants with the salicylate mimic, benzothiadiazole: (1) attenuates the jasmonate-induced expression of the antiherbivore defense-related enzyme polyphenol oxidase, and (2) compromises host-plant resistance to larvae of the beet armyworm, *Spodoptera exigua.* Conversely, treatment of plants with jasmonic acid at concentrations that induce resistance to insects reduces pathogenesis-related protein gene expression induced by benzothiadiazole, and partially reverses the protective effect of benzothiadiazole against bacterial speck disease caused by *Pseudomonas syringae* pv. tomato. We conclude that effective utilization of induced plant resistance to the multiple pests typically encountered in agriculture will require understanding potential signaling conflicts in plant defense responses.

Key Words—Benzothiadiazole, salicylic acid, jasmonic acid, defense signaling.

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INTRODUCTION

Plant defense against pathogens and insects is mediated in part by an array of constitutive and inducible chemical resistance factors (Duffey and Felton, 1989; Bennett and Wallsgrove, 1994). Some defenses against insects may be effective against attackers from diverse kingdoms including bacterial and fungal pathogens (McIntyre et al., 1981; Karban et al., 1987; Stout et al., 1998), while other defenses are specific to particular attackers (Carroll and Hoffman, 1980; Apriyanto and Potter, 1990; Krischik et al., 1991; Ajlan and Potter, 1992; Giamoustaris and Mithen, 1995). Since defenses effective against all challengers are absent, plants must coordinate multiple defense strategies that may have different effects on different pests (Linhart, 1991). Distinct responses to pathogens and herbivores appear to be highly conserved in plants (Herrmann et al., 1989; Choi et al., 1994; Schneider et al., 1996). Studies of systemic acquired resistance (SAR) to pathogens and induced resistance (IR) to insects have proceeded with little consideration of possible interactions between the two (Karban and Baldwin, 1997; Durner et al., 1997). There has been considerable interest in developing chemicals to induce the biochemical pathways in economically important plants that provide resistance to either pathogens or insects. An understanding of how pathways controlling SAR and IR interact will be critical for using chemical inducers to provide effective pest management.

Salicylic acid is a key compound in a pathway that regulates resistance to fungal, bacterial, and viral pathogens and provides a signal for expression of pathogenesis-related (PR) proteins and other potentially protective factors induced following pathogen challenge (Enyedi et al., 1992; Ryals et al., 1996). Jasmonic acid, produced by the octadecanoid pathway via lipoxygenation of linolenic acid, serves as a signal for expression of a number of proteins including polyphenol oxidase (PPO) and proteinase inhibitors (PI), proteins that appear to contribute to plant resistance against many insect attackers (Broadway et al., 1986; Farmer et al., 1992). Salicylic acid, the potent synthetic salicylate mimic benzothiadiazole-7-carbothioic acid *S*-methyl ester (BTH), and jasmonic acid (JA) provide pure chemical inducers of SAR or IR that can be exogenously applied to plants to stimulate many of the systemic metabolic changes that follow challenge with pathogens or insects (Ryals et al., 1992; Kessmann et al., 1994; Gorlach et al., 1996; Thaler et al., 1996).

There is evidence from laboratory studies for interference between the salicylate and octadecanoid signaling pathways (Doherty et al., 1988), whereby salicylic acid appears to inhibit jasmonic acid biosynthesis and the subsequent chemical responses (Pena-Cortes et al., 1993; Doares et al., 1995). There is also evidence for inhibition of salicylate action by jasmonic acid (Sano and Ohashi, 1995). This negative interaction between the two response pathways, previously demonstrated at the biochemical level, may compromise the ability of plants to

coordinate defense against simultaneous challenge from pathogens and herbivores typically encountered in field settings.

In this report, we provide evidence from a field experiment for a tradeoff between salicylate-induced resistance to the bacterial speck pathogen, *Pseudomonas syringae* pv. tomato, and jasmonate-induced resistance to the noctuid caterpillar, *Spodoptera exigua.* Both are common pests of tomato plants in California (Strand and Rude, 1998). We found that treating plants with compounds that engage the two pathways separately resulted in the induction of the appropriate chemical responses and biological resistance to challenge insects and pathogens previously reported for each pathway (Fidantsef et al., 1999; Stout et al., 1999). However, when the plants are sprayed to stimulate both response pathways simultaneously, the corresponding biological effects on resistance to the pathogen and the insect are compromised.

METHODS AND MATERIALS

Plant Growth and Elicitor Treatment. The experiments used 4-week-old tomato plants grown in the field using standard agricultural practices. The plants were divided among four treatment groups in a two-way factorial design with BTH and JA as factors: (1) plants sprayed with BTH (1.2 mM BTH, approx. 3.0 μmol per plant) to stimulate SAR, (2) plants sprayed with JA (1.0 mM JA, approx. 2.5 μmol per plant) to stimulate IR, (3) plants sprayed simultaneously with BTH and JA (approx. 3.0 μmol BTH, 2.5 μmol JA), and (4) control plants sprayed with the inert carrier of the commercial BTH formulation. JA was suspended as an emulsion in water and acetone by brief sonication. Fifteen single plant replicates of each treatment were used for each chemical assay, and 30 single plant replicates were used for each bioassay.

Chemical Assays. Five days after the plants were treated, we measured the levels of chemical indicators of each pathway to determine the level of activation of the salicylate- and jasmonate-mediated responses. We measured mRNA levels of the tomato PR protein, P4, as a marker of BTH action, and PPO and PI activity as markers for jasmonic acid action. The fourth youngest leaf of 15 plants from each treatment was collected for biochemical analysis six days after the plants were sprayed. The terminal leaflet was used for the PPO assay, and the remainder of the leaf was used to obtain RNA for gel blot analyses. PPO activity was determined according to the methods described elsewhere (Thaler et al., 1996). Briefly, PPO was extracted by homogenizing weighed leaflets in ice-cold buffer, then centrifuged to obtain a clarified extract. Fifteen microliters of the supernatant was added to a caffeic acid solution (2.92 mM in pH 8 potassium phosphate buffer) and the increase in OD at 470 nm of the mixture was measured spectrophotometrically.

Accumulation of mRNA for the tomato PR protein P4 and for the proteinase inhibitor PINII was determined by analysis of total RNA prepared from tomato leaves (Choi et al., 1994). RNA (20 μg/lane) was fractionated by electrophoresis through 1% agarose gels containing formaldehyde and transferred to Nytran membranes. The insert from a plasmid vector containing a sequence for P4 was amplified by PCR using commercial primers, T3 and T7. The PCR products were separated in 0.7% agarose (FMC, Inc.) in TAE buffer. The target fragments were excised from the gel using the Qiaex Gel Extraction Kit (Qiagen, Chatsworth, California) according to the manufacturer's directions. PINII (Graham et al., 1985) was obtained by digestion of the plasmid vector pT2-47 containing the tomato PINII with EcoRI and HindIII, which produced a fragment of about 700 bp. The DNA probes were radioactively ([^{32}P]CTP) labeled with the Random Primed DNA Labeling Kit (Boehringer Mannheim, Indianapolis, Indiana) following the manufacturer's protocol. The blots were hybridized with the *pinII* probe, and then stripped and hybridized with the *p4* probe. Hybridized blots were stripped by placing them three times, 15 min each time, in a boiling solution containing 2 mM PB, 0.025 mM EDTA, 0.005% NaPPi, and 0.05% SDS. Hybridization and wash conditions were those as described by Ghosh and Kopp (1995) for P4 and by Graham et al. (1985) for PINII. The amount of radioactively labeled probe hybridized to each RNA sample (20 μg) was estimated with a two-dimensional radioisotope imaging system (Ambis, Inc., San Diego, California). Equal loading of RNA samples was verified by hybridization of the blots with a tomato cyclophilin DNA probe and by staining of rRNAs with ethidium bromide.

Bioassays. A suspension of *P. syringae* pv. tomato cells corresponding to 1×10^7 colony CFU/ml was applied gently with a paint brush to the upper surface of the terminal leaflet of the fourth leaf of each plant. The number of bacterial speck lesions per inoculated leaflet was determined five days after inoculation. Five *S. exigua* neonates were placed on each plant and the plants enclosed within a light-admitting spun polyester mesh cage (Kleen Test Products, Brown Deer, Wisconsin). Larvae were allowed to feed on the plants for 10 days, after which the surviving larvae on each plant were counted and weighed. Thirty replicates of each treatment were established for each experiment.

Herbivore Abundance. A set of plants was sprayed once early in the season but not subsequently challenged. The numbers of naturally occurring western flower thrips (*Frankliniella occidentalis*) were counted on the terminal leaflet of all leaves three weeks after the treatments were applied.

Plant Yield. Fruits were harvested when 10% of the fruit had turned pink. At this time, fruits were counted, weighed, and divided into groups according to quality. The groups were clean, sunburn, stinkbug damage, small dry wound, small wet wound, and large wet wound. These are factors used commercially to assess fruit quality.

Statistical Analyses. Plant yield data were analyzed using MANOVA with BTH and JA treatments as independent factors and fruit quantity and fruit quality measures as the dependent factors. All other data were analyzed using two-way ANOVA with BTH and JA treatments as the independent factors. The number of larvae surviving per plant was log-transformed for analysis so that our hypothesis fit a multiplicative risk model (Sih et al., 1998).

RESULTS

Chemical Analyses. We found that mRNA levels of the pathogenesis-related (PR) protein P4 were higher, but not statistically significant, in the plants sprayed with BTH and lower in plants sprayed with JA compared to control plants, as shown in Figure 1 (two-way ANOVA: for BTH, $F_{1,52} = 3.5$, $P = 0.07$; for JA, $F_{1,52} = 4.6$, $P = 0.04$; or BTH * JA interaction, $F_{1,52} = 1.6$, $P = 0.16$). The plants sprayed with both BTH and JA had P4 transcript levels similar to the

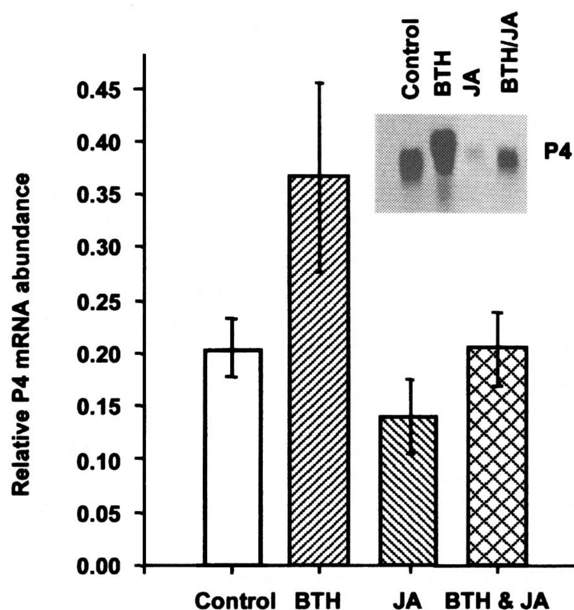

FIG. 1. Pathogenesis-related protein P4 mRNA abundance in inert carrier-treated plants (control) or in plants treated with BTH, JA, or BTH and JA. Abundance is expressed relative to the maximum level of transcript abundance detected with the probe in one sample. Means ± SE are shown. Inset: Representative blot of the levels of P4 transcripts obtained in the four treatments.

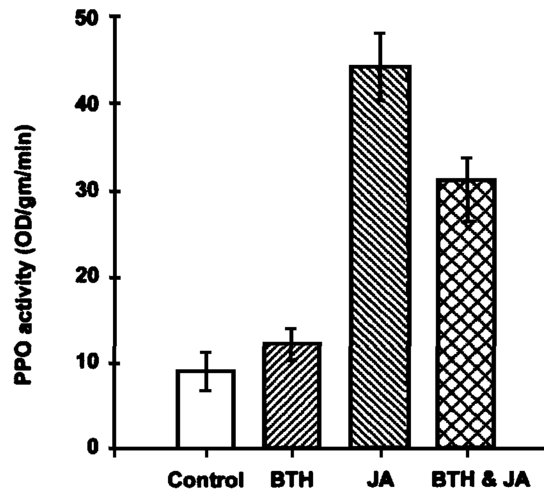

FIG. 2. Polyphenol oxidase activity (Δ OD/gm/min) measured in the terminal leaflet of the fourth youngest leaf of plants treated with inert carrier (control) or with BTH, JA, or BTH and JA.

controls, with the individual effects of BTH and JA apparently canceling each other out. PPO activities were highest in the plants sprayed with JA and lowest both in the plants sprayed with BTH and the control plants (Figure 2, two-way ANOVA: for BTH, $F_{1,56} = 1.2$, $P = 0.28$; for JA, $F_{1,56} = 63.2$, $P < 0.001$; for BTH * JA interaction, $F_{1,56} = 5.6$, $P = 0.02$). The plants sprayed with both BTH and JA had intermediate PPO activities compared with the plants treated separately. The statistical interaction between JA and BTH indicates that their effects on PPO levels were not additive; BTH alone does not affect PPO activity, but in the presence of JA, BTH reduces PPO activity.

PINII mRNA accumulation generally reflected the same pattern in response to the treatments as did PPO activity. PINII mRNA levels were highest in JA-treated plants and lowest in plants sprayed with BTH (mean relative mRNA levels ± SE in control: 0.146 ± 0.065, BTH: 0.079 ± 0.068, JA: 0.278 ± 0.062, BTH/JA: 0.032 ± 0.060; two-way ANOVA for BTH, $F_{1,42} = 5.9$, $P = 0.019$; for JA, $F_{1,42} = 0.4$, $P = 0.51$; for BTH * JA interaction, $F_{1,42} = 1.9$, $P = 0.17$). Unlike PPO, the effect of JA on PINII mRNA was not statistically significant above the control.

Bioassays. Differences in PR protein mRNA levels and PPO activity correlated with the subsequent performance of *P. syringae* pv. tomato and *S. exigua* on challenged leaves. Plants sprayed with BTH had the fewest bacterial speck lesions, whereas the plants sprayed with JA and controls had the most lesions

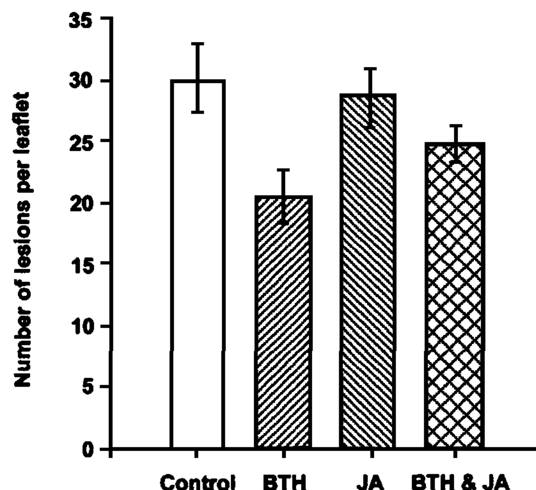

FIG. 3. The number of bacterial speck lesions on inert carrier-treated plants (control) or on plants treated with BTH, JA, or BTH and JA.

(two-way ANOVA: for BTH, $F_{1,114} = 8.9$, $P = 0.003$; for JA, $F_{1,114} = 0.3$, $P = 0.2$; for BTH * JA interaction, $F_{1,114} = 1.7$, $P = 0.19$; Figure 3). The lack of statistical interaction indicates that the effects of JA and BTH on lesion number were additive. The plants sprayed with both BTH and JA had an intermediate number of lesions, but this was not significantly different from the control or BTH-sprayed plants. This result could have been caused by JA attenuation of BTH action.

Survivorship (Figure 4) and weight of *S. exigua* were affected by the treatments. JA reduced and BTH enhanced survival of the *S. exigua* larvae (two-way ANOVA for BTH, $F_{1,111} = 4.4$, $P < 0.001$; for JA, $F_{1,111} = 12.1$, $P < 0.001$; for BTH * JA interaction, $F_{1,111} = 0.25$, $P = 0.29$). The lack of statistical interaction indicates that the effects of JA and BTH on survivorship were additive. Insects feeding on plants in the BTH/JA treatment had intermediate survivorship. The greater survivorship of *S. exigua* on BTH plants compared with control plants may have reflected the greater ability of control plants to activate resistance mechanisms against the assay larvae. In contrast, the BTH plants may not have been able to activate resistance because BTH may interfere with JA synthesis and action. Alternatively, unrelated responses activated by BTH may have increased leaf suitability to herbivores. While both BTH and JA influenced larval survivorship, only JA influenced larval weight (mean weight mg ± SE in control: 5 ± 1, in BTH: 5 ± 1, in JA: 2 ± 0.5, BTH/JA: 2 ± 0.5; two-way ANOVA for BTH, $F_{1,79} = 0.1$, $P = 0.7$; for JA, $F_{1,79} = 7.4$, $P = 0.008$; for BTH * JA interaction, $F_{1,79} = 1.4$, $P = 0.8$). The mean

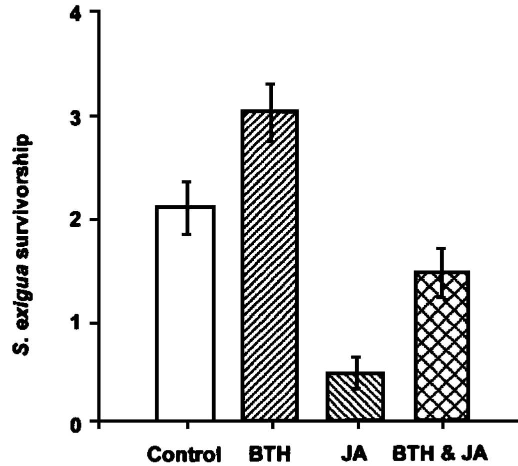

FIG. 4. The number of surviving *S. exigua* larvae per plant on inert carrier-treated plants (control) or on plants treated with BTH, JA, or BTH and JA. Untransformed data are presented.

weights of the surviving larvae from JA- and JA/BTH-sprayed plants were half of those from BTH-sprayed and control plants.

Naturally Occurring Herbivores and Plant Yield. We determined the abundance of naturally occurring herbivores and the quantity and quality of fruits produced by plants in our four treatment groups. The number of naturally occurring thrips was decreased by the JA and BTH/JA treatment but was not affected by BTH in comparison to the controls (Figure 5, two-way ANOVA for BTH,

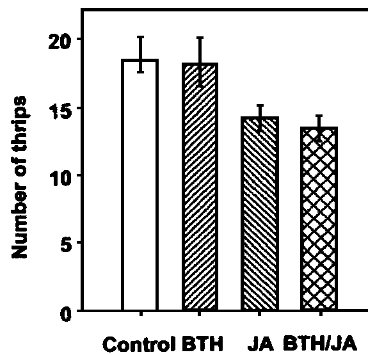

FIG. 5. The number of thrips on inert carrier-treated plants (control) or on plants treated with BTH, JA, or BTH and JA.

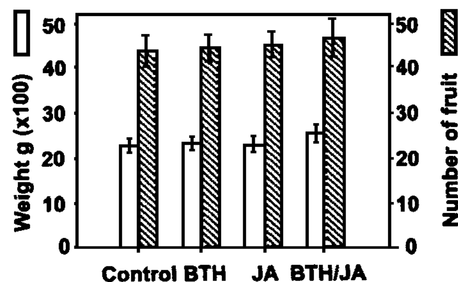

FIG. 6. The number of fruit and weight of fruit collected from inert carrier-treated plants (control) or from plants treated with BTH, JA, or BTH and JA.

$F_{1,116} = 0.01$, $P = 0.9$; for JA, $F_{1,116} = 16.4$, $P < 0.001$; for BTH * JA interaction, $F_{1,116} = 0.4$, $P = 0.5$). End of season yield (Figure 6) and fruit quality (data not shown) were not affected by any of the spray treatments (MANOVA: $F_{28,361} = 0.5$, $P = 0.9$).

DISCUSSION

Our results provide evidence for reciprocal attenuation of signaling pathways that produce IR to an insect herbivore and SAR to a bacterial plant pathogen in tomato plants under field conditions. The trade-off in mobilization of defense responses operates in both directions. The expression patterns of defense-related indicators suggest that at least a portion of the trade-off is due to biochemical interference of signal–response pathways. However, the trade-off was not observed for thrips abundance. There was no effect on overall plant yield.

Salicylic acid applied exogenously to tomato plants will inhibit wound-induced proteinase inhibitor synthesis (Doherty et al., 1988; Pena-Cortes et al., 1993) possibly through the inhibition of jasmonate biosynthesis and action (Doares et al., 1995). This effect may be explained in part by the capacity for salicylate and related hydroxybenzoates to interact with Fe^{2+} in heme- and non-heme-containing proteins, either through chelation or as an electron donor to generate a highly reactive salicylate radical (Ghosh and Kopp, 1995; Rüffer et al., 1995; Durner et al., 1997). For example, a salicylic acid binding protein was characterized in tobacco as a catalase (Chen et al., 1993), and allene oxide synthase (Pan et al., 1998) is a step in jasmonate synthesis that appears to be a site for salicylate inhibition. Both are heme proteins.

SAR and IR have been associated with pathogen and herbivore attack, respectively. This strict dichotomy is an oversimplification, however, when plant

responses to diverse attackers are considered (Li et al., 1996; Pieterse et al., 1998). In the tomato system, some herbivores induce SAR and some pathogens induce IR. For instance, *Macrosiphum euphorbiae* (insect) feeding induces PR proteins, markers of SAR, and *Pseudomonas syringae* (pathogen) attack induces PIs, markers of IR, in addition to SAR (Fidantsef et al., 1999). In order to understand the generality of the trade-off between plant resistance to herbivores and pathogens, we need to examine which pathways are induced by a broader range of pathogens and insects and which organisms are affected by elicitation of these pathways (Stout et al., 1998, 1999; Thomma et al., 1998; Vijayan et al., 1998).

This interference between defenses effective against herbivores and pathogens poses a potential problem in disease and insect management. Since crop plants typically are challenged by multiple parasites at the same time, it may not be possible to simultaneously maximize defense against all kinds of attackers. In a natural environment, an advantage of inducible defenses over constitutively expressed defenses may be to allow the plant to engage signal-response pathways that will be most effective against the respective challengers. Inhibition of jasmonate-mediated signaling by salicylates might also interfere with the attraction by wounded plants of the natural enemies of herbivores (Dicke et al., 1990; Turlings et al., 1990; Drukker et al., 1995; Shimoda et al., 1997). Recent work has shown that production of volatile parasitoid attractants is regulated by the octadecanoid (JA) pathway (Alborn et al., 1997, Thaler, 1999). It will be important to consider the trade-offs occurring when host plants encounter multiple stresses typical of field settings during the development of new strategies that incorporate inducible resistance mechanisms for pathogen and insect management.

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MECHANISMS OF HYDROQUINONE-INDUCED GROWTH REDUCTION IN LEAFY SPURGE

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Abstract—Field observations indicate leafy spurge (*Euphorbia esula*) is inhibited by the presence of *Antennaria microphylla.* Hydroquinone (HQ), one of several compounds isolated from *A. microphylla* has been shown to inhibit leafy spurge seed germination, root elongation, and callus culture growth. The present study was designed to analyze the effects of HQ on water relations and photosynthesis of leafy spurge. Plants grown in 0.25 mM HQ had consistently higher leaf diffusive resistance and lower transpiration rates than control plants ($P < 0.05$). Chlorophyll fluorescence was significantly lower than controls ($P < 0.05$) towards the end of the treatment period. At the end of the treatment, tissue from 0.25 mM HQ plants had higher levels of ^{13}C, indicating there had been a sustained interference with stomatal function. These data suggest that a disruption of the plant water balance is one mechanism of leafy spurge inhibition by *A. microphylla.*

Key Words—Hydroquinone, allelopathy, plant water balance, photosynthesis, chlorophyll fluorescence, ^{13}C isotopes, leafy spurge, *Euphorbia esula*, small everlasting, *Antennaria microphylla* Rydb.

INTRODUCTION

Leafy spurge (*Euphorbia esula* L.) is a Eurasian plant species that was introduced onto the North America continent in the late 1800s. Since that time,

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leafy spurge has become well established and currently infests extensive areas of rangeland throughout much of the northern Great Plains. In North Dakota alone, leafy spurge infestations reduced the carrying capacity of grazeable land, impacting regional economies at an annual cost of about US$75 million (Leistritz et al., 1992). Much of the weed control effort focuses on conventional control/eradication programs utilizing herbicides, an approach that is expensive and therefore limited to areas of high productivity. Alternative approaches to control leafy spurge include biological insect control (Gagne, 1990; Gassman and Shorthouse, 1990; Pecora et al., 1989, 1991) and encouraging lambs and goats to graze preferentially on leafy spurge (Walker et al., 1992).

Leafy spurge dominates and displaces many native plant species in a variety of habitats. Belcher and Wilson (1989) found that leafy spurge significantly decreased the frequency of five common native mixed-grass species in Manitoba, Canada. Within portions of the North Dakota Badlands, native grasslands deteriorate further when heavy infestations of leafy spurge cause shifts in habitat utilization by native ungulates (Trammel and Butler, 1995). That research demonstrates that bison, elk, and mule deer shift their foraging away from areas of leafy spurge infestation and towards remaining noninfested areas. Increased grazing of these noninfested areas could facilitate further encroachment of leafy spurge.

While many native plants are displaced by leafy spurge, small everlasting (*Antennaria microphylla* Rydb.), a common native forb, is phytotoxic to and resists encroachment by leafy spurge (Selleck, 1972). It is unlikely that competition alone explains the inhibition of leafy spurge due to the fact that the plant is a deep rooted vigorous clonal species, whereas small everlasting is shallow rooted and grows relatively slowly. In the field, small everlasting inhibited the growth of leafy spurge while soil removed from around small everlasting had a similar effect (Selleck, 1972). In this same study, plant and soil extracts of small everlasting inhibited leafy spurge seed germination and seedling development. Extraction and isolation of compounds from small everlasting yielded hydroquinone (HQ), arbutin (a monoglucoside of HQ), caffeic acid, and benzoquinone (Manners and Galitz, 1985). This experiment also demonstrated that HQ was the most phytotoxic of the extracted compounds to seed germination and root elongation of leafy spurge. In another study, it was shown that media extracts from callus cell cultures of small everlasting and exogenously supplied HQ inhibited both leafy spurge callus tissue and suspension culture growth (Hogan and Manners, 1990).

Allelopathy refers to the process by which certain compounds (allelochemicals) produced by plants are released into the environment where they can interfere with the growth of other plants. In direct contrast to competition, allelopathic interactions involve the addition of chemical substances into the environment. It has been well documented that interference by certain donor species,

particularly by the release of phenolic compounds, is related to disruption of certain physiological processes of target plants (Einhellig, 1986; 1995). The allelopathic influence of HQ on the growth of soybean has been linked to changes in plant water relations (Barkosky, 1988). One primary mechanism of interference may be a perturbation of cell membranes, which would likely influence plant water relations and lead to a reduction in overall plant growth (Einhellig, 1986, 1995). Determination of intermediate or secondary physiological effects, including interactions with plant hormones, ion uptake, mitochondrial respiration, and photosynthesis, could lead to further insight into the mode of action of allelochemicals.

The focus of this investigation was to study the allelopathic mechanism of action of HQ on leafy spurge by relating any changes in growth to effects on water relations and photosynthesis.

METHODS AND MATERIALS

Lateral root sections of leafy spurge, collected from a local infestation near Vermillion, South Dakota, were transplanted into 4-quart pots filled with commercial potting soil and allowed to regrow. Cuttings were made by following the procedure outlined below that was developed by Lyn (1992) and modified by Barkosky (1997). Stem tips 75–80 mm long were cut from individual plants and all but two or three of the uppermost leaves removed. The cut end was then dipped in commercial root hormone and placed into vermiculite moistened with reverse-osmosis water. After two days, cuttings were transferred to individual 400 ml opaque vials filled with 0.5 strength Hoagland's nutrient solution for 28 days. HQ treatments began on day 31 by transferring cuttings into nutrient medium amended with HQ, and solutions were replaced every three days throughout the treatment period to ensure consistent nutrient levels and exposure to HQ. In a preliminary 14-day experiment, plants were treated with 0.25, 0.5, and 0.75 mM HQ in order to determine the amount of HQ needed for growth inhibition. From this experiment it was determined that 0.25 mM HQ was the lowest concentration that significantly reduced the growth of leafy spurge within this system. Plants were then treated for a longer duration (30 days) using 0.25 and 0.1 mM HQ.

Plants were exposed to greenhouse conditions throughout the propagation and treatment period. Baseline diffusive leaf resistance and transpiration readings were taken the day before treatment began (day 30 of the propagation period) and then every three days between 12:00 and 15:00 hr throughout the treatment period using a Li-Cor 1600 steady-state porometer. Measurements were made on all six plants per treatment group on the abaxial surface of three of the newest leaves per plant that were sufficiently large to completely fill the

porometer cuvette. Chlorophyll fluorescence was chosen to evaluate the effects of HQ on photosynthesis and was measured on the same three leaves chosen for measurement with the porometer. Krause and Weis (1991) summarized the evidence and method showing chlorophyll fluorescence to be a valuable tool for measuring PSII efficiency. Baseline fluorometer readings were obtained the day before treatment began and then every six days during the experiment using an Opti-Sciences-modulated fluorometer. Leaves were dark-adapted for 15 min prior to illumination with the fluorometer. Data are expressed as the F_v/F_m ratio, a value that is an important measure of the physiological state of the photosynthesis activity.

At the end of the experiment, plants were harvested by removing leaves from the plant to obtain leaf area. All tissue was oven-dried at 105°C for 72 hr. Leaf area was determined by first photocopying all leaves from each plant and then using a Panasonic Video Digitizer with Digital Image Analysis System computer software (Decagon Device, Inc., Pullman, Washington). Leaf tissue was analyzed for carbon isotope ratio ($^{13}C:{}^{12}C$) with an isotope-ratio mass spectrometer at the Duke University Phytotron, Durham, North Carolina. Samples were prepared for analysis by grinding oven-dried leaves in a Cyclone Sample Mill fitted with a 0.4-mm screen. Results of this analysis are expressed as a $\delta^{13}C$ value obtained from equation 1 (O'Leary, 1988).

$$\delta^{13}C\% = \frac{{}^{13}C/{}^{12}C_{(\text{sample})} - {}^{13}C/{}^{12}C_{(\text{standard})}}{{}^{13}C/{}^{12}C_{(\text{standard})}} \times 1000 \qquad (1)$$

The effect of HQ on growth and carbon isotope discrimination was analyzed by using one-way analysis of variance (ANOVA) with means separated by Duncan's multiple range test. Porometer and fluorometer data were analyzed using a repeated measures design with treatment (HQ) and day of treatment (0–30) as independent variables. Sidak's t test was used to compare experimental groups. All analyses were conducted using the Statistical Analysis System (SAS Institute 1992).

RESULTS

Leafy spurge cuttings exposed to 0.5 mM and 0.75 mM HQ in the 14-day preliminary experiment exhibited stunted growth and had visible signs of damage including chlorotic looking or dead older leaves, dark purple younger leaves, and black slimy roots. Although growth reduction occurred in plants in the 0.25 mM HQ group, they did not exhibit the same visible signs of damage as did plants subjected to higher concentrations. Plants in all treatment groups had

TABLE 1. EFFECT OF HYDROQUINONE (HQ) ON GROWTH OF LEAFY SPURGE AFTER 14-DAY TREATMENT[a]

	HQ treatment			
Plant variable	Control	0.25 mM	0.5 mM	0.75 mM
Leaf area (cm^2)	113 (5) a	76 (4) b	70 (2) bc	64 (3) c
Leaf wt. (mg)	354 (26) a	227 (16) b	157 (8) c	144 (10) c
Root wt. (mg)	58 (5) a	27 (4) b	17 (2) b	26 (6) b
Shoot wt. (mg)	223 (18) a	139 (9) b	132 (9) b	115 (6) b
Plant wt. (mg)	635 (4) a	393 (3) b	306 (1) b	285 (2) c

[a]Mean (SE) values ($N = 6$) within rows not followed by the same letter are significantly different ($P < 0.05$), ANOVA with Duncan's multiple range test.

significantly lower leaf area, leaf weight, root weight, and shoot weight when compared to control plants (Table 1).

Data from the preliminary experiment established 0.25 mM HQ as the concentration that would inhibit growth of leafy spurge, but also allow leaves to develop to sufficient size for measurement with the porometer and fluorometer over a 30-day treatment period. At the end of the 30-day experiment, both the 0.1 mM and 0.25 mM HQ treatments reulted in significant reductions in growth when compared with controls (Table 2). At both treatment levels, all growth variables measured were significantly lower than control values, and all but shoot weight were significantly lower between the 0.1 and 0.25 mM HQ treatment groups.

Beginning early in the second week of the experiment (day 9), leafy spurge

TABLE 2. EFFECTS OF HYDROQUINONE (HQ) ON GROWTH OF LEAFY SPURGE AFTER 30-DAY TREATMENT[a]

	HQ treatment level		
Plant variable	Control	0.10 mM	0.26 mM
Leaf area (cm^2)	289 (10) a	204 (4) b	90 (7) c
Leaf wt. (mg)	985 (48) a	710 (46) b	371 (24) c
Root wt. (mg)	225 (11) a	164 (15) b	88 (6) c
Root length (cm)	23 (1) a	12 (1) b	7 (1) c
Shoot wt. (mg)	903 (102) a	384 (62) b	259 (8) b
Shoot length (cm)	43 (2) a	29 (1) b	20 (1) c
Plant wt. (mg)	2113 (80) a	1258 (119) b	718 (33) c

[a]Mean (SE) values ($N = 6$) in a row not followed by the same letter are significantly different ($P < 0.05$), ANOVA with Duncan's multiple range test.

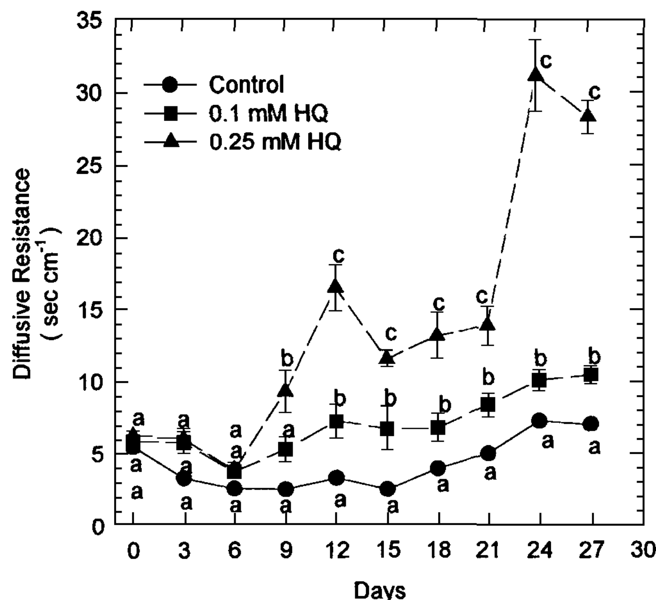

FIG. 1. Effect of hydroquinone (HQ) on leaf diffusive resistance in leafy spurge over 30 days of treatment. Each value is the mean of six plants; different letters indicate significance between groups, a_c = 0.03, with Sidak's t test.

plants treated with 0.25 mM HQ had significantly higher diffusive resistance than controls (Figure 1). These high resistances continued throughout the duration of the treatment period. By day 12, plants in both treatment groups had significantly higher diffusive resistance than controls (Figure 1). Following a similar pattern, transpiration rates of treated plants were significantly lower than control plants at both treatment levels by the third day of treatment (Figure 2).

Photosynthesis activity in the 0.25 mM treated plants began to decline below that of controls starting in the third week of the experiment, as indicated by the F_v/F_m ratio (Figure 3). In the last week of treatment these plants also had significantly lower F_v/F_m values than plants treated with 0.1 mM HQ. Plants treated at the lower concentration of HQ also had significantly lower F_v/F_m ratios than controls by the end of the treatment period (Figure 3).

Treatment for 30 days with 0.25 mM HQ resulted in plants that had discriminated less against ^{13}C over the duration of the experiment, as indicated by a less negative $\delta^{13}C$ (Table 3). Plants treated with 0.1 mM HQ had $\delta^{13}C$ values similar to controls (Table 3).

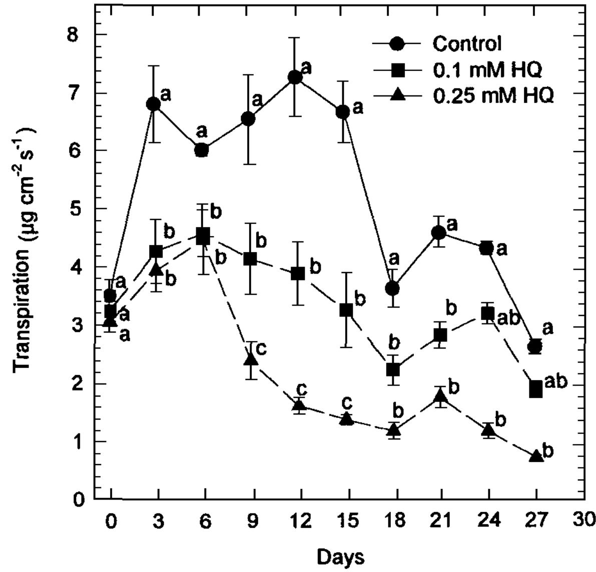

FIG. 2. Effect of hydroquinone (HQ) on transpiration in leafy spurge over 30 days of treatment. Each value is the mean of six plants; different letters indicate significance between groups, $a_c = 0.03$, with Sidak's test.

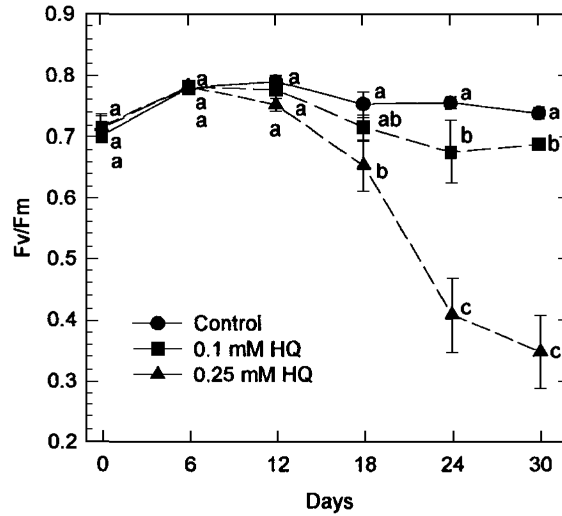

FIG. 3. Effect of hydroquinone (HQ) on chlorophyll fluorescence in leafy spurge over 30 days of treatment. Each value is the mean of six plants; different letters indicate significance between groups, $a_c = 0.03$, with Sidak's t test.

TABLE 3. EFFECT OF HYDROQUINONE (HQ) ON STABLE CARBON ISOTOPE RATIOS IN LEAFY SPURGE[a]

	HQ treatment		
Plant parameter	Control	0.10 mM	0.25 mM
$\delta^{13}C$ (%)	−28.14 (0.10) a	−28.16 (0.16) a	−25.81 (0.09) b

[a]Mean (SE) values ($N = 6$) in a row not followed by the same letter are significantly different ($P < 0.05$), ANOVA with Duncan's multiple range test.

DISCUSSION

These experiments clearly demonstrate that exposure to as little as 0.1 mM HQ inhibits the growth of leafy spurge at the whole plant level. Furthermore, given the close parallel between effects of growth on water status, a disruption in water relations appears to be the primary mode of action that leads to reductions in overall growth. In past research, changes in water relations have been associated with the allelopathic action of several plants. Extracts of *Kochia scoparia* have been shown to increase leaf diffusive resistance and reduce leaf water potential in soybean and sorghum (Einhellig et al., 1982). In a related study, extracts of velvetleaf (*Abutilon theophrasti* Medic.) increased diffusive resistance and lowered leaf water potential and relative water content in soybean (Colton and Einhellig, 1980). In earlier work, we determined that treatment with 0.25 mM HQ increased stomatal resistance, lowered transpiration, and reduced the growth of soybean seedlings (Barkosky, 1988). Data from the current experiment show that leafy spurge exposed to either treatment levels experienced changes in stomatal function early in the experiment as evidenced by high resistances and low transpiration rates. As the experiment progressed, it is likely that this water stress led to the decline in the chlorophyll fluorescence of treated plants.

The F_v/F_m ratio, typically 0.75–0.85 for nonstressed plants, is highly correlated with the quantum yield of net photosynthesis (Araus and Hogan, 1994). A reduction in F_v/F_m can either indicate damage to thylakoid membranes, particularly those associated with PSII reaction centers, or inhibition of excitation energy transfer from antenna to reaction centers (Krause and Weis, 1984). It has been shown that disruption of photosynthetic activity can occur when a vapor pressure deficit-induced stomatal closure reduces transpiration and uptake of CO_2 (Ben et al., 1987). It is also possible that, in this particular experiment, a chronic reduction in the amount of available CO_2 during the 30-day exposure to HQ resulted in lower F_v/F_m values for treated plants.

Diffusional limitation of CO_2 through the stomata decreases discrimination against the heavy isotope, while factors influencing enzyme activity tend to

increase the discrimination (Berry, 1989; Farquhar et al., 1989; O'Leary, 1988). The carbon isotope ratios of plants treated with 0.25 mM HQ are consistent with stomatal diffusion limitations. If HQ had negatively impacted photosynthetic activity directly (e.g., reducing the activity of photosynthetic enzymes or damaging thylakoid membranes), then the carbon isotope ratio would have likely shown more discrimination against ^{13}C and chlorophyll fluorescence may have declined earlier in the treatment period. Plants treated with 0.1 mM HQ did exhibit some changes in stomatal function and overall growth compared to untreated plants, but the effects were much less than the results in leafy spurge treated with 0.25 mM HQ. Likewise, the 0.1 mM HQ plants did not discriminate against ^{13}C differently than controls.

Although there is no direct evidence from this experiment, changes in water balance of treated plants could have been initiated at the site of action, the root/treatment solution interface, by inhibition of ion uptake across root plasma membranes. It has been suggested that perturbations of cell membranes by phenolic compounds may cause physiological changes that combine to reduce plant growth (Einhellig, 1986, 1995). In this model, membrane perturbations represent the "the common denominator" in an array of physiological changes that reduce plant growth. Glass and Bohm (1971) demonstrated that HQ interferes with $^{86}Rb^{+}$ ion uptake in barley roots, suggesting a disruption of active transport. It is possible that disruption of leafy spurge root membranes led to the physiological changes observed in this experiment by inhibiting ion and water uptake. According to Einhellig (1986, 1995), allelochemicals can alter membrane permeability by disrupting structural associations, modifying membrane channels, and reducing the activity of membrane-bound carrier proteins. Baziramakenga et al. (1995) reported that the benzoic and cinnamic acids caused changes in the content of sulfhydryl groups within membrane proteins. Any of these membrane effects would affect ion transport and water relations and could ultimately influence stomatal function and photosynthesis.

It is also possible that direct disruption of stomatal function, at the level of guard cell membranes, could account for the observed changes in plant water balance and that this ultimately affected ion and water uptake. Perhaps HQ directly influenced guard cell membranes by a similar mechanism, as noted above for perturbation of root membranes, which then led to stomatal closure. It has also been shown that allelochemicals can affect ABA levels. ABA is a phytohormone that has a regulatory role in stomatal function and so influences water relations (Holappa and Blum, 1991).

This experiment documents some of the phytotoxic effects of HQ on the physiology and growth of leafy spurge. This and other experiments tend to support the field observation that small everlasting resists encroachment by leafy spurge. In the absence of a recognized ecological interaction, this experiment would simply represent a bioassay-type experiment using leafy spurge as the

test species. A bioassay of this type could be useful in a program to produce naturally occurring herbicides, but would perhaps lack ecological validity. Furthermore, the foundation of this and other experiments lies with the premise that HQ is released in sufficient quantities by small everlasting to inhibit the growth of leafy spurge. The most likely avenue of release is by way of seasonal decomposition of small everlasting, although rot exudation may play a role. The argument also could be made that other factors, such as interspecific competition, are responsible for the observed interference, but in light of the pronounced morphological dichotomy between the two species, the relative contributions of both competition and allelopathy have to be determined to fully understand the interaction.

In conclusion, these experiments demonstrate that HQ inhibits the growth of leafy spurge and that a disruption of plant water relations may be an important mode of action that leads to the reduction in growth.

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TASTE RESPONSIVENESS TO FOOD-ASSOCIATED ACIDS IN THE SQUIRREL MONKEY (*Saimiri sciureus*)

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Abstract—The taste responsiveness of six adult squirrel monkeys to five food-associated acids was assessed in two-bottle preference tests of brief duration (5 min). In experiment 1 the monkeys were given the choice between tap water and defined concentrations of citric acid, ascorbic acid, malic acid, acetic acid, or tannic acid dissolved in tap water. In experiment 2 the animals were given the choice between a 50 mM sucrose solution and defined concentrations of the same acids dissolved in a 50 mM sucrose solution. With both procedures *Saimiri sciureus* significantly discriminated concentrations as low as 10 mM ascorbic acid and acetic acid, 5 mM citric acid and malic acid, and 0.2 mM tannic acid from the alternative stimulus. The results showed: (1) that squirrel monkeys respond to the same range of acid concentrations as other nonhuman primate species tested so far, (2) that *Saimiri sciureus* detects food-associated acids at concentrations well below those present in most fruits, and (3) that the responsiveness of this New World primate to acidic tastants was largely unaffected by the addition of a sweet-tasting substance. The results support the assumption that squirrel monkeys may use sourness and/or astringency of food-associated acids as a criterion for food selection.

Key Words—Gustatory preference thresholds, taste sensitivity, acidic compounds, squirrel monkey, *Saimiri sciureus*.

INTRODUCTION

The taste of most fruits is characterized by a mixture of sensations termed as sweet and sour by humans (Nagy and Shaw, 1980), and the food selection behavior of nonhuman primates suggests that they may use gustatory cues, and the relative salience of sweetness and sourness in particular, to assess palatability and nutritional value of a fruit (Clutton-Brock, 1977; Glaser, 1989). Thus, high sensitivity allowing the detection of both sweet- and sour-tasting substances should be important for frugivorous primates.

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While the sense of taste has been investigated behaviorally and electrophysiologically in a number of primate species, most studies so far have concentrated on detectability of the four basic taste qualities, usually using acetic acid as the only, prototypic, sour stimulus. There is only sparse information as to the taste responsiveness of nonhuman primates for naturally occurring acids other than acetic acid.

Fruits usually contain a variety of acidic tastants (Ulrich, 1970), although ascorbic acid, citric acid, and malic acid are quantitatively predominant in most cases and may account for more than 90% of total acid content (Souci et al., 1989). Ascorbic acid plays essential roles in several physiological functions in mammals. Whereas many species of mammals synthesize their ascorbate requirements, all primate species examined so far are unable to do so (Milton and Jenness, 1987), and thus primates are thought to require a dependable dietary supply of ascorbic acid (Portman, 1970).

In addition to carbohydrates, citric acid and malic acid are the most common and abundant means of storing respiratory energy in fruits (Ulrich, 1970), and they represent an easily metabolizable source of energy for frugivorous primates (Gallina and Ausman, 1979). Furthermore, citric acid is known to act as an antirachitic agent in primates because it facilitates the absorption of calcium (Portman, 1970).

Acetic acid is also widely distributed in fruits, although usually only at low concentrations (Ulrich, 1970). However, it is a main product of fermentation, putrefying, and oxidation processes of plant material and thus is a potential indicator of the degree of ripeness of a fruit and concomitantly of its nutritional value (Lang, 1970).

Tannic acid also is present in a wide spectrum of plant matter, particularly in foliage, the skin and husks of fruits, and the bark of trees (Swain, 1979). It binds protein and amino acids and can prevent their absorption (Goldstein and Swain, 1965). Therefore, it seems adaptive for monkeys to be able to taste tannic acid in order to select food sources without too much of this substance. In humans, tannic acid elicits a characteristic astringent and—at high concentrations—bitter taste (Lyman and Green, 1990), and in rhesus monkeys tannic acid has been shown to be a major determinant of food selection (Marks et al., 1988).

Given the presumed importance of food-associated acids for food selection in primates, it was the aim of the present study to assess the gustatory responsiveness of squirrel monkeys—a frugivorous New World primate species—to ascorbic acid, citric acid, malic acid, acetic acid, and tannic acid, all important constituents of the natural diet in *Saimiri sciureus*.

Because most, but not all primate species tested so far reject substances that taste sour and/or astringent to humans (Glaser and Hobi, 1985), a two-bottle preference test of brief duration (5 min) was employed. This methodology makes it possible to directly measure preferences for or aversions to tastants and

largely rules out the influence of postingestive factors on the animal's ingestive behavior. Tap water (experiment 1) or a sucrose solution (experiment 2), both as solvent for the acids and as the alternative stimulus, allowed me to additionally address the question of whether adding a sweet-tasting substance would affect the monkeys' responsiveness to the acids.

METHODS AND MATERIALS

Animals. Testing was carried out with three male and three female adult squirrel monkeys (*Saimiri sciureus*) weighing 0.8–1.2 kg and ranging from 4 to 8 years of age. This social group was housed in a 10-m^3 enclosure, which could be subdivided by sliding doors to allow temporary separation of animals for individual testing, and was maintained on a 12L:12D cycle at 20–24°C. Animals were fed marmoset pellets (Ssniff) and fresh fruit and vegetables ad libitum but were deprived of water overnight before testing on the following morning. The amount of food offered daily to the animals was such that leftovers were still present on the next morning and thus it was unlikely that ravenous appetite affected the animals' ingestive behavior in the tests.

Procedure. Taste responsiveness to ascorbic acid, citric acid, malic acid, acetic acid, and tannic acid (reagent grade, Merck) was assessed in a two-bottle preference test. Twice each day, approximately 3 and 1 hr before feeding, the animals were separated and allowed 5 min to drink from a pair of simultaneously presented graduated 100-ml cylinders with metal drinking tubes.

In experiment 1, the monkeys were given a choice between tap water and defined concentrations of the acids dissolved in tap water. In experiment 2, the monkeys were given a choice between a 50 mM sucrose solution and defined concentrations of the acids dissolved in a 50 mM sucrose solution. In both experiments testing usually started at a concentration of 100 mM and proceeded in the following steps: 50, 20, 10, 5, 2, 1 mM, etc., until the animals failed to show a significant preference or aversion. With tannic acid, testing started at a concentration of 5 mM and proceeded in the same manner as for the other substances. Higher concentrations were additionally tested with ascorbic acid (400 and 200 mM) and acetic acid (200 mM) in order to evaluate whether such an increase in stimulus intensity would lead to an even more pronounced behavioral response.

Each pair of stimuli was presented 10 times, and the position of the stimuli was randomized in order to counterbalance possible position preferences. All animals had served in previous studies with the same methodology (Laska, 1994, 1996, 1997) and were completely accustomed to the procedure.

The experiments reported here comply with the "Principles of Animal Care" (publication No. 86-23, revised 1985 of the NIH) and also with current German laws.

Data Analysis. For each animal, the amount of liquid consumed from each bottle was recorded, summed for the 10 test trials with a given stimulus combination, converted to percentages (relative to the total amount of liquid consumed from both bottles), and 66.7% (i.e., 2/3 of the total amount of liquid consumed) was taken as criterion of preference. This rather conservative criterion was chosen for reasons of comparability of data because the same criterion had been used in previous studies with the same methodology and individuals (Laska, 1994, 1996, 1997), and in order to avoid misinterpretation of data due to a too liberal criterion.

Additionally, two-tailed binomial tests (Siegel and Castellan, 1988) were performed and an animal was only regarded as significantly preferring one of the two alternative stimuli if it reached the criterion of 66.7% and consumed more from the bottle containing the preferred stimulus in at least 8 of 10 trials (binomial test, $P < 0.05$).

Preliminary analysis of the data indicated that there were no reliable differences in choice behavior and liquid consumption between the male and female subjects and between the first and the second presentation of the day. Intraindividual variability of the amount of liquid consumed across the 10 test trials with a given stimulus combination was low and averaged less than 20%. Thus, a theoretically possible bias in the overall preference score due to excessive drinking in aberrant trials did not occur. Therefore, the data for the males and females obtained in the 10 test trials were combined and reported as group means with standard deviations.

Preliminary tests showed that the animals rejected the solutions containing detectable concentrations of the acids. Following convention, the results are nevertheless expressed as percentage of preference for the tastant and not for the solvent. Accordingly, 33.3% (i.e., 1/3 of the total amount of liquid consumed) was taken as criterion of avoidance.

RESULTS

Experiment 1. Figure 1 shows the mean performance of the six squirrel monkeys in the two-bottle preference tests with tap water used both as solvent for the acids and as the alternative stimulus. All six animals significantly discriminated concentrations as low as 10 mM ascorbic acid and acetic acid, 5 mM citric acid and malic acid, and 0.2 mM tannic acid from tap water, and in some cases single individuals even scored slightly lower values. All animals rejected detectable concentrations of all acids tested and in no case showed a preference for an acid. However, all animals failed to reject the lowest concentrations presented, suggesting that the avoidance of higher concentrations was indeed based on taste perception. In most cases, interindividual variability of scores was low for both sub- and suprathreshold concentrations tested (cf. SDs in Figure 1).

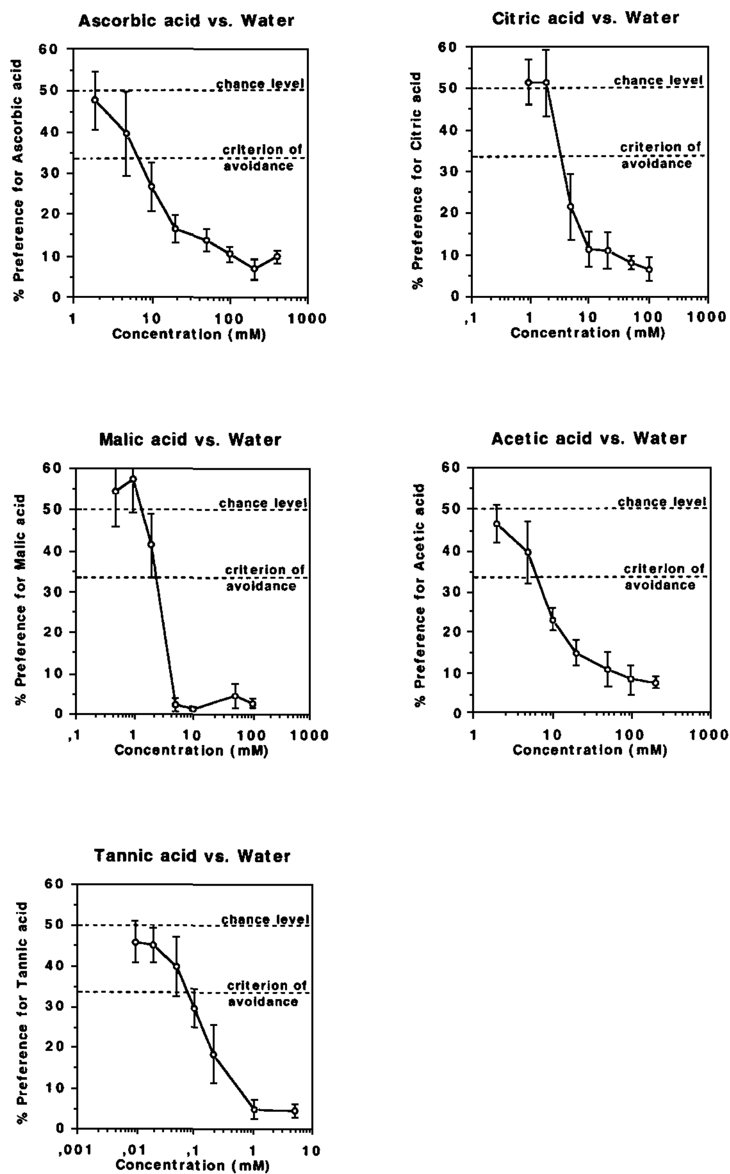

FIG. 1. Taste responsiveness of six squirrel monkeys to ascorbic acid, citric acid, malic acid, acetic acid, and tannic acid dissolved in tap water and tested against tap water as alternative stimulus. Each point represents the mean value (±SD) of 10 test sessions of 5 min per animal.

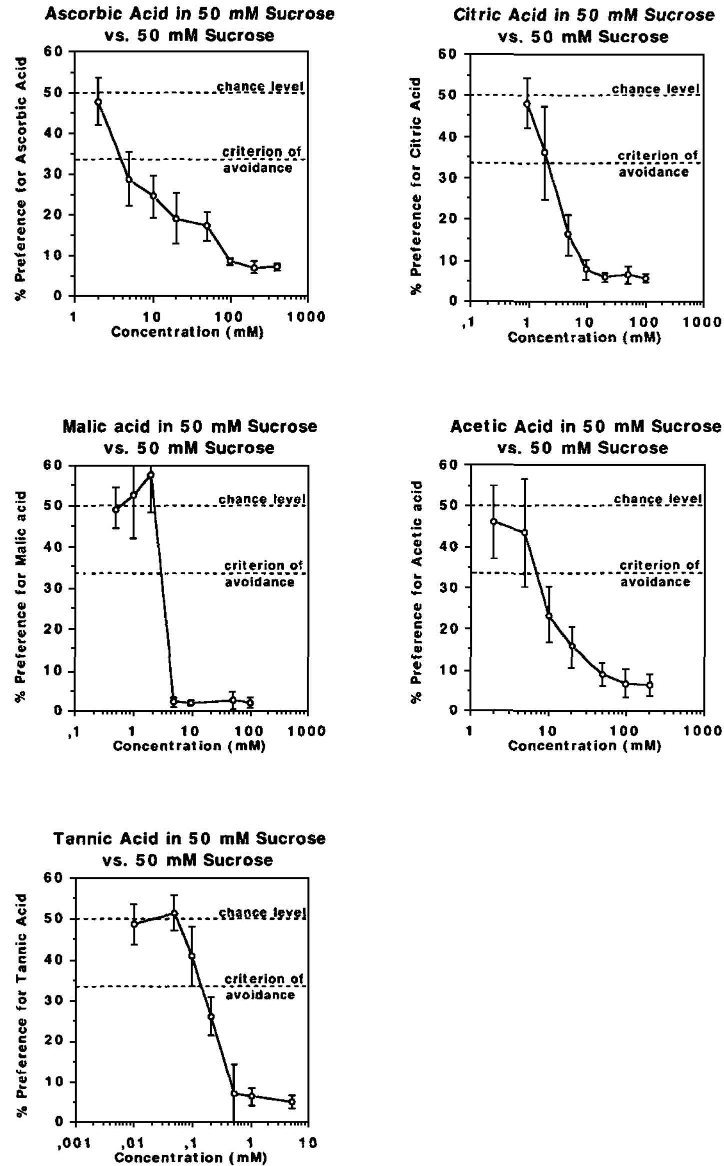

FIG. 2. Taste responsiveness of six squirrel monkeys to ascorbic acid, citric acid, malic acid, acetic acid, and tannic acid dissolved in a 50 mM sucrose solution and tested against a 50 mM sucrose solution as alternative stimulus. Each point represents the mean value (±SD) of 10 test sessions of 5 min per animal.

Experiment 2. Figure 2 shows the mean performance of the six squirrel monkeys in the two-bottle preference tests with a 50 mM sucrose solution used both as solvent for the acids and as the alternative stimulus. In accordance with the findings from experiment 1, all six animals significantly discriminated concentrations as low as 10 mM ascorbic acid and acetic acid, 5 mM citric acid and malic acid, and 0.2 mM tannic acid from the alternative stimulus, and again in some cases single individuals even scored slightly lower values. Despite the sweet-tasting—and thus highly attractive—solvent used in this experiment, all acids were rejected at detectable concentrations by all animals. The lowest acid concentrations presented, however, were consumed at equal amounts compared to the alternative stimulus, suggesting that the avoidance of higher concentrations was indeed based on perception of the acidic tastants. In most cases, interindividual variability of scores was low as can be inferred from the SDs in Figure 2.

DISCUSSION

The results of this study can be regarded as a first and conservative approximation of the gustatory sensitivity squirrel monkeys for food-associated acids. Table 1 compares the taste responsiveness of *Saimiri sciureus* obtained here with the few data available from other primate species. Squirrel monkeys responded to the same range of concentrations as other nonhuman primates tested so far. The finding that the preference thresholds found here were only about one order of magnitude higher than the detection thresholds established in humans (ASTM, 1973) is remarkable considering that the sophisticated psychophysical signal detection methods employed with human subjects are likely to be more sensitive compared to the simple two-bottle preference test used with the monkeys. This supposition is supported by findings from Pritchard et al. (1995), who reported taste thresholds obtained with a conditioning paradigm in *Macaca mulatta* to be lower than those obtained with a preference test. This suggests that the sensitivity of *Saimiri sciureus* for food-associated acids might indeed match, if not exceed, that of *Homo sapiens*.

The reliability of the data reported here is supported by the fact that very similar values for citric acid and acetic acid were obtained with a single squirrel monkey in an earlier study (Glaser and Hobi, 1985) and the same methodology but a slightly different criterion.

The acid concentrations detected by the squirrel monkeys are well below those present in most fruits (Souci et al., 1989) or other parts of plants that are consumed by this species (Albach et al., 1981; Milton and Jenness, 1987). Thus, it seems reasonable to assume that the sourness and/or astringency elicited by the acids in their natural diet enter into the food selection behavior of *Saimiri sciureus*.

TABLE 1. TASTE RESPONSIVENESS TO FOOD-ASSOCIATED ACIDS IN SQUIRREL MONKEYS AND OTHER PRIMATE SPECIES

	Conc. (mM)				
Species[a]	Ascorbic acid	Citric acid	Malic acid	Acetic acid	Tannic acid
Saimiri sciureus[1]	10	5	5	10	0.2
Microcebus murinus[2,5]				8	*0.2*
Loris tardigradus[2]				17	
Nycticebus coucang[2]				6	
Galago senegalensis[2]				4	
Cebuella pygmaea[2]		6		6	
Saguinus midas niger[2]		4		4	
Aotus trivirgatus[2]		4		8	
Saimiri sciureus[2]		8		8	
Ateles geoffroyi[3]	5				
Macaca fascicularis[6]	*10*	*10*	*10*	*10*	*10*
Homo sapiens[4]	1	0.4	0.4	0.8	0.006

[a]Behavioral thresholds: [1]present study, [2]Glaser (1986), [3]Laska et al. (unpublished), and [4]ASTM (1973). *Electrophysiological responses:* [5]Hellekant et al. (1993) and [6]Plata-Salaman et al. (1995).

At detectable concentrations, all acids tested were rejected by the squirrel monkeys. This behavioral response is in accordance with reports of their natural feeding behavior (Baldwin, 1985) and with most of the few experimental studies that presented other primate species with acidic tastants (Glaser, 1986). So far, only one New World primate species, the owl monkey (*Aotus trivirgatus*), has been reported to prefer citric acid and acetic acid over tap water at low but detectable concentrations (Glaser and Hobi, 1985), and thus to show an inverted U-shaped function of preference, which also has been described in humans and some nonprimate mammal species (Kare, 1971). The authors hypothesize this preference for acidic tastants as an evolutionary adaptation of this species to avoid competition pressure between sympatric frugivores for sweet-tasting fruits.

A final aspect of the present study is the finding that the responsiveness of *Saimiri sciureus* to acidic tastants was largely unaffected by the addition of a sweet-tasting substance. The preference threshold values obtained in both experiments were identical and the degree of rejection at suprathreshold concentrations did not differ markedly between experiments 1 and 2, suggesting that the monkeys perceived an acid similarly when dissolved in tap water or in a sucrose solution. Numerous psychophysical studies have shown that both the intensity and the quality of a taste stimulus, as perceived by humans, is modified by including that stimulus in a mixture (McBride, 1989). However, there are conflicting findings with regard to the nature and degree of this modification: Suppression and

enhancement of one tastant by another have been reported to occur in the perception of taste mixtures (Gregson and McGowen, 1963), although the former seems to be the rule (Schifferstein and Frijters, 1993). In a recent study, Stevens (1996) reported that sucrose has only a small masking effect on citric acid in humans, a finding which is in accordance with the responsiveness shown by the squirrel monkeys.

Considering that frugivorous species must make complex decisions in order to select a nutritionally balanced and nontoxic diet from among the available plants that may vary greatly in their chemical composition, it makes sense for such a species to be able to detect constituents of a potential food that presumably are critical for food choice as well in a taste mixture as when presented singly. This idea concurs with electrophysiological recordings from gustatory neurons in the primary taste cortex of the cynomolgus monkey (*Macaca fascicularis*) that have shown sour-best cells to be less susceptible to mixture suppression than, for example, sugar-best cells (Plata-Salaman et al., 1996).

Taken together, the results of the present study support the assumption that squirrel monkeys may use sourness and/or astringency of food-associated acids as a criterion for food selection.

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RESPONSES OF JAPANESE GIANT LOOPER MALE MOTH TO SYNTHETIC SEX PHEROMONE AND RELATED COMPOUNDS

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Abstract—The Japanese giant looper, *Ascotis selenaria cretacea*, is a serious defoliator of tea gardens in Japan. In Mie Prefecture male moths were dose-dependently attracted to rubber septa baited with 0.1–10.0 mg of the sex pheromone component (Z,Z)-6,9-*cis*-3,4-epoxynonadecadiene. Monitoring by pheromone traps showed three flight periods (June, July, and August–September). A septum with a 1-mg dose was still active after exposure for over three months in the field, but the number of males captured by the old lure was one fifth of that captured by a fresh lure. The parent 3,6,9-triene, a minor pheromonal component, showed a synergistic effect on male attraction when mixed with the pheromonal epoxydiene at a very low ratio, but the 1 : 1 mixture hardly captured any male moths. Attraction was also effectively inhibited by the positional isomers (6,7- and 9,10-epoxydienes), which exhibited rather strong electrophysiological activities on the male antennae. When these related compounds were placed around a trap baited with the synthetic pheromone, the number of captured moths remarkably decreased, which indicates the possibility of using the compounds as a disruptant.

Key Words—Sex pheromone, attractant, Japanese giant looper, *Ascotis selenaria cretacea*, (Z,Z)-6,9-*cis*-3,4-epoxynonadecadiene, field attractant, disruption, electroantennogram.

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INTRODUCTION

The sex pheromones of lepidopterous defoliators in tea gardens have been studied in Japan. Monitoring techniques and direct population control by disruption of their mating communication have been established for the smaller tea tortrix (*Adoxophyes* sp.) and the Oriental tea tortrix (*Homona magnanima* Diakonoff) with synthetic pheromone components (Wakamura, 1992). In Mie and Shizuoka Prefectures, the Japanese giant looper (mugwort looper), *Ascotis selenaria cretacea* Butler (Geometridae: Ennominae), also seriously damages tea leaves. We are eager to establish similar monitoring and disruption techniques for *A. s. cretacea* in order to cultivate tea plants with limited use of insecticides. The sex pheromone of the giant looper distributed in Israel, *Ascotis (Boarmia) selenaria* Denis & Schiffermüller, was identified as the 3*S*,4*R* isomer of (*Z*,*Z*)-6,9-*cis*-3,4-epoxynonadecadiene, (epo3,*Z*6,*Z*9–19:H) (Becker et al., 1983, 1990; Cossé et al., 1992), and recently we detected the same *cis*-epoxide in the pheromone gland extract of the Japanese subspecies (Ando et al., 1997). While both enantiomers were observed in this extract, the 3*R*,4*S* isomer interestingly showed stronger attractive activity than the racemic mixture, suggesting that the two subspecies are reproductively isolated from each other not only geographically but also by pheromonal communication. This paper deals with the results of the field tests conducted for evaluating synthetic lures baited with the racemic mixture of epo3,*Z*6,*Z*9–19:H as a monitoring tool of *A. s. cretacea*. We also measured the electroantennogram (EAG) response of several pheromonal analogs. Furthermore, inhibitory activity of some analogs against the sex pheromone component was examined in order to examine the possibility of their utility as a disruptant.

METHODS AND MATERIALS

Chemicals. 3,6,9-Trienes and their monoepoxides were synthesized starting from linolenic acid and purified by a Lobar column (Merck LiChroprep Si 60) (Ando et al., 1993, 1995). Chemicals with a purity of >97% and >99% on a capillary GC (DB-23, 0.25 mm ID × 30 m, J & W Scientific) were used for field experiments and EAG tests, respectively.

Field Evaluation of Synthetic Lures. The chemical dissolved in *n*-hexane (150 μl) was impregnated into a gray rubber septum and the lure was placed on the center of a sticky trap (30 × 27-cm bottom plate with a roof, Takeda Chemical Ind., Ltd., Osaka, Japan), which was placed 1.0 m above the ground in tea gardens from 1996 to 1997. Population monitoring was simultaneously examined in two tea gardens at Yokkaichi-shi and Kameyama-shi in Mie Prefecture separated by a distance of ca. 20 km using one trap for each garden. An other

evaluation of the synthetic pheromone was carried out at one of the gardens with three traps for each lure, except for a test of the 10-mg dose, in which two traps were used. The attractive activity of each treatment was evaluated by counting the captured male moths.

Disruption Experiments. To conduct the disruption experiment, wires holding a disruptant were attached around the Takeda sticky trap, i.e., upward, downward, and forward of two windows of the trap. The investigated disruptant dissolved in *n*-hexane (150 μl) was placed into four gray rubber septa; then the dispensers were connected to the ends of the wires, 20 cm from the center of the trap baited with epo3,Z6,Z9–19:H (1 mg). Tests were conducted in Kameyama-shi in 1997 with three traps for each experiment, and disruption efficiency was calculated by dividing the difference between the numbers of the males captured by the control and test traps by the former.

EAG Test. Larvae of *A. s. cretacea* were reared on a semisynthetic diet for the silkworm [Insecta LF(S), Nippon-Nosan-Kogyo Co., Yokohama, Japan] at 20°C on a 16L:8D cycle, and the 2- or 3-day-old male moths were used for EAG studies. This electrophysiological assay was carried out by modifying the method as described in the review by Roelofs (1984). Each test material (10 μg) was applied to a small piece of paper (1 × 2 cm) fixed in a 5-ml glass syringe. After removing the head of the male moth and placing it on tissue paper wetted with insect saline, 1 ml of the air containing the chemical vapor was puffed on one of two antennae connected to a recording electrode. The responses were recorded with a recorder (model 8K30, Nippondenki-Sanei, Co., Tokyo, Japan) instead of a cathode ray oscilloscope.

RESULTS

Population Monitoring by Pheromonal Epoxydiene. A. s. cretacea passes the winter in the pupal stage. First adult emergence from hibernating pupae is generally observed from May to early June in Mie Prefecture. After that, two more adult flights in July and late August to September usually occur. Using a trap baited with epo3,Z6,Z9–19:H (1 mg/septum) and renewing the lure each month, we captured and counted males every five days in 1996. In total, 506 and 359 males were attracted in Yokkaichi-shi and Kameyama-shi, respectively, and the results of the one-year survey indicate three generations (Figure 1). Although a peak of first flight was recorded rather later than early June in Yokkaichi-shi, the three flight periods observed in Kameyama-shi are in good agreement with those of the previous data investigated by light trap for forecasting.

Active Period of Dispenser and Dose Response. After exposing dispensers baited with epo3,Z6,Z9–19:H (1 mg/septum) under field conditions during several periods (0, 17, 36, 68, and 103 days), the lures were put in the traps placed

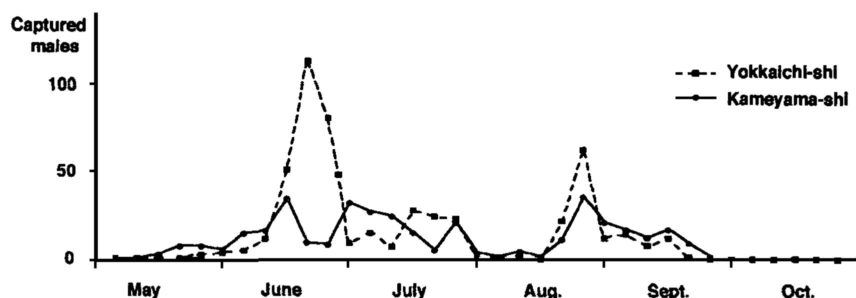

FIG. 1. Monitoring of *A. s. cretacea* flights by a sticky trap baited with pheromonal epoxydiene (epo3,Z6,Z9–19 : H) in tea gardens in Mie Prefecture.

in a tea garden in Yokkaichi-shi. We compared their attractiveness during the third flight period of *A. s. cretacea* in 1996 in order to evaluate the active period of the synthetic lures. Table 1 lists the average number of males captured by one trap per day. The 1-mg lure was still active after more than three months in the field, although the activity diminished during exposure and the number of males captured by the oldest lure was one fifth of that captured by the lure most recently fixed in the traps.

The amount of synthetic lure applied to a rubber septum is another important factor that affects trap efficiency. We examined several dosages of epo3,Z6,Z9–19 : H in 1996 and 1997. The results of these experiments are shown in Table 2, which indicates that the lure dose has a positive correlation with the number of trapped male moths. Activity of the 1.0-mg lure was strongest among

TABLE 1. ATTRACTION OF *Ascotis selenaria cretacea* IN TEA GARDENS BY RUBBER SEPTA BAITED WITH SYNTHETIC PHEROMONE (epo3,Z6,Z9–19:H), EXPOSED IN A FIELD FOR DIFFERENT PERIODS

Lure (1 mg/septum)		Captured males
Start of exposure	Test period after exposure	(N/trap/night)[a]
Aug. 31	days 0–15	5.24 a
Aug. 14	days 17–32	4.13 a
July 25	days 36–51	2.53 a
June 24	days 68–83	1.56 ab
May 20	days 103–118	1.09 b

[a]Average number of captured males by three traps in Mie Prefecture (Yokkaichi-shi) from Aug. 31 to Sept. 15, 1996. Values followed by a different letter are significantly different at $P < 0.05$ by Duncan's multiple range test.

TABLE 2. FIELD ATTRACTION OF *Ascotis selenaria cretacea* IN TEA GARDENS IN MIE PREFECTURE (YOKKAICHI-SHI) BY TRAPS WITH DIFFERENT DOSES OF SYNTHETIC PHEROMONE epo3,Z6,Z9–19:H

	Average of captured males (*N*/trap/night)[a]		
Treatment (mg/septum)	Aug. 22–Sept. 8, 1996	June 10–19, 1997	Aug. 16–22, 1997
10.0[b]		5.94 a	10.42 a
5.0[c]		4.33 a	7.94 a
1.0[c]	3.18 a	0.78 b	2.39 b
0.5[c]	1.47 a		
0.1[c]	0.39 ab		
0.0[c]	0.00 b	0.00 b	0.00 c

[a]Values within each column followed by a different letter are significantly different at $P < 0.05$ by Duncan's multiple range test.
[b]Captured by two traps.
[c]Captured by three traps.

the 0.1- to 1.0-mg doses tested in 1996, and weakest among the 1.0- to 10.0-mg doses tested in 1997.

Effect of Related Compounds Mixed with Pheromonal Epoxydiene. Field attraction of synthetic epo3,Z6,Z9–19 : H (1 mg/septum) mixed with (Z,Z,Z)-3,6,9-nonadecatriene (Z3,Z6,Z9–19 : H), the parent triene (0.01–1.0 mg/septum), or one of the positional isomers, Z3,epo6,Z9–19 : H and Z3,Z6,epo9–19 : H (0.1–1.0 mg/septum), was separately measured in different tea gardens, and the results are given in Table 3. A small amount of the triene (0.01 mg/septum) unambiguously exhibited a synergistic effect on the attraction of *A. s. cretacea*. Increasing its quantity, however, resulted in decreasing numbers of captured males, and epo3,Z6,Z9–19 : H was almost completely inactive when the same amount of triene (1.0 mg/septum) was present in the same dispenser. Two positional isomers also inhibited attraction activity of the pheromonal epoxydiene at the dose ranges tested. Almost no male moths were caught by traps baited with 1 : 1 mixtures of epo3,Z6,Z9–19 : H and positional isomers. Among the three tested compounds, Z3,epo6,Z9–19 : H exhibits the strongest inhibitory activity. In this field test, a noctuid species, *Hypersypnoides submarginata*, was attracted to the mixtures of epo3,Z6,Z9–19 : H and Z3,Z6,Z9–19 : H.

Disrupting Activity of Pheromonal Epoxydiene and Related Compounds. Table 4 shows field attraction of *A. s. cretacea* to traps surrounded with disruptants. The placement of a dispenser with 0.2 mg of Z3,Z6,Z9–19 : H; Z3,epo6,Z9–19 : H; or Z3,Z6,epo9–19 : H around the trap baited with pheromonal epoxydiene (1.0-mg dispenser of epo3,Z6,Z9–19 : H) effectively reduced the number of captured males in mid-September when the population density was

TABLE 3. FIELD ATTRACTION OF *Ascotis selenaria cretacea* IN TEA GARDENS IN MIE PREFECTURE BY epo3,Z6,Z9–19:H (1 mg/SEPTUM) MIXED WITH RELATED COMPOUNDS

Mixed compound dose (mg/septum)	Average of captured males (N/trap/night)[a]			
	Z3,Z6,Z9–19:H[b]			
	Test I	Test II	Z3,epo6,Z9–19:H[c]	Z3,Z6,epo9–19:H[c]
1.0	0.15 d	0.00 d	0.04 b	0.02 b
0.5	1.11 c	0.61 d	0.06 b	0.17 b
0.1	2.85 b	3.39 c	2.52 a	2.74 a
0.05	6.63 ab	8.00 b		
0.01	8.30 a	14.39 a		
0.00	4.30 b	5.83 bc	3.64 a	3.64 a

[a]Values within each column followed by a different letter are significantly different at $P < 0.05$ by Duncan's multiple range test.
[b]From June 10 to 19 (test I) and from Aug. 16 to 22 (test II), 1997, in Yokkaichi-shi. In test II, lures mixed with 1.0, 0.5, 0.1, and 0.05 mg of the triene captured 6, 8, 9, and 3 males of *Hypersypnoides submarginata* Walker (Noctuidae: Ophiderinae), respectively.
[c]From Aug. 11 to Sept. 16, 1997, in Kameyama-shi.

TABLE 4. FIELD ATTRACTION OF *Ascotis selenaria cretacea* BY TRAPS POSSESSING ATTRACTANT (epo3,Z6,Z9–19:H, 1 mg/SEPTUM) AND DISRUPTANTS IN TEA GARDEN IN MIE PREFECTURE (KAMEYAMA-SHI).

Dose of disruptant (mg/septum)	Average of captured males [N/trap/night (Disruption, %)][a]			
	epo3,Z6,Z9–19:H	Z3,Z6,Z9–19:H	Z3,epo6,Z9–19:H	Z3,Z6,epo9–19:H
Test I (Sept. 11–16, 1997)				
5.0		0.00 (100)	0.00 (100)	0.00 (100)
1.0	4.53	0.27 (89)	0.07 (97)	0.53 (78)
0.2		0.67 (72)	0.47 (80)	1.33 (45)
0.0 (control)		2.40		
Test II (Sept. 18–29, 1997)				
5.0		0.00 (100)	0.00 (100)	0.00 (100)
1.0	1.54	0.00 (100)	0.00 (100)	0.00 (100)
0.2		0.00 (100)	0.36 (35)	0.27 (51)
0.0 (control)		0.55		

[a]Disruption (%) was calculated as $[(N_c - N_t)/N_c] \times 100$, where N_c and N_t are numbers caught by the control and test traps, respectively.

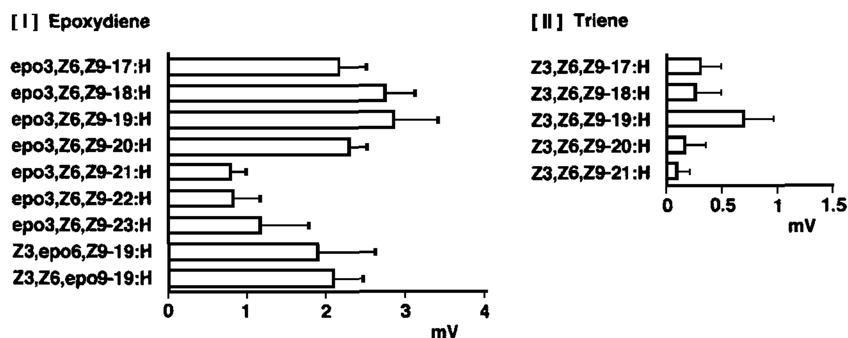

FIG. 2. EAG responses of *A. s. cretacea* males to the pheromonal epoxydiene (epo3,Z6,Z9–19 : H), the parent hydrocarbon (Z3,Z6,Z9–19 : H), and their related compounds.

rather high, and no males were captured by the placement of 5.0 mg of disruptants. Among the three compounds, the highest disruption was accomplished by Z3,epo6,Z9–19 : H. In the field with low densities in late September, even 1.0 mg of disruptant completely suppressed the activity of the synthetic lure. The placement of 1.0 mg of disruptant composed of the pheromonal epoxydiene, however, caused an increase in captured males.

EAG Responses. Figure 2 has the EAG profiles of *A. s. cretacea* males. The pheromonal epo3,Z6,Z9–19 : H showed the strongest activity in a series of 3,4-epoxy compounds. The antennae also moderately responded to the positional isomers, Z3,epo6,Z9–19 : H and Z3,Z6,epo9–19 : H. The response to Z3,Z6,Z9–19 : H was weak but stood out above other tested trienes.

DISCUSSION

Monitoring pest populations with a pheromone-baited trap has long been recognized as one of the major benefits of sex pheromone research. The pheromone trap offers several advantages over other methods of sampling populations. First, such a trap is relatively inexpensive and easy to deploy. Second, it generally catches only the target species. Results of field tests carried out in Mie Prefecture confirmed that the synthetic pheromonal epoxydiene in a racemic form (epo3,Z6,Z9–19 : H) was a useful tool for monitoring the adult flight of *A. s. cretacea*, whose larva is one of the serious defoliators in tea gardens. The eggs hatch one week after laying, and ca. 40 days are needed for full growth at 20°C; thus we can predict the optimum time for spraying an insecticide. While the high-dose lure (10 mg/septum) showed stronger attractive activity than any other lure, the activity of the 1-mg lure was acceptable. After setting the moni-

toring trap in late spring, renewing the lure two or three times may be sufficient for a one-year survey in Japan.

In addition with epo3,Z6,Z9–19 : H, we have identified the parent triene (Z3,Z6,Z9–19 : H) in the pheromone gland extract of *A. s. cretacea*. The ratio of the epoxide and triene was less than 1 : 0.05 during scotophase and was converted to 1 : 1 at the end of photophase. This triene is a biosynthetic precursor of the epoxydiene, and its synergistic effect was not observed in our previous field tests (Ando et al., 1997). Females of *A. selenaria* in Israel, the subspecies of *A. s. cretacea*, also produce Z3,Z6,Z9–19 : H, but the triene was not found in the volatiles of the calling female, and it decreased behavioral responses of males to the epoxy lure in a wind tunnel (Cossé et al., 1992). In the present tests, we could confirm a synergistic effect of the triene when it was mixed with the epoxydiene in a very low ratio. Furthermore, male antennae of *A. s. cretacea* responded to the triene. These results indicate that the triene is an important minor component for sexual communication of the Japanese species, although it has not been resolved whether the triene actually occurs in the volatiles of *A. s. cretacea.*

Many sex pheromones of geometrid females are composed of 3,6,9-trienes, 6,9-dienes, and their monoepoxy derivatives with a C_{17}–C_{23} straight chain (Arn et al., 1992, 1997). Although chemical structural variations are limited, there are about 800 species of geometrid moths distributed in Japan, and isomers of the pheromonal epoxydiene, Z3,epo6,Z9–19 : H and Z3,Z6,epo9–19 : H, are expected to be pheromone components of some other geometrid species. These latter compounds exhibited rather high EAG activity and strongly inhibited the attractiveness of epo3,Z6,Z9–19 : H when mixed in a ratio higher than 1 : 0.5. The 1 : 0.1 mixtures, however, showed similar activity to the unmixed pheromonal epoxydiene, indicating that closely related species might not utilize epo3,Z6,Z9–19 : H as a major component and the positional isomers as a minor component.

We synthesized the pheromonal epoxydiene via the oxidation of Z3,Z6,Z9–19 : H by a peracid. This epoxidation proceeded unselectively and yielded a mixture of three epoxydienes. The requisite epo3,Z6,Z9–19 : H could be separated by medium-pressure liquid chromatography (Ando et al., 1995), but it is difficult to prepare a pure sample. Fortunately, male moths responded to the lure contaminated with a small amount of the positional isomers.

McDonough et al. (1994) evaluated communication disruption by synthetic codlemone and its isomers that were impregnated in septa and placed around a trap for the codling moth (*Cydia pomonella* L.). In our experiments, utilizing similarly modified traps, attraction of epo3,Z6,Z9–19 : H was inhibited by Z3,Z6,Z9–19 : H and positional isomers of the pheromonal epoxydiene that were placed 20 cm from the lure. The pheromonal epoxydiene itself did not act as a disruptant. Pheromone components have been selected as optimum chemicals to disrupt the chemical communications between lepidopterous females and males

(Wakamura, 1992). We observed disruption of the cabbage looper (*Mamestra brassicae* L.) and the diamondback moth (*Plutella xylostella* L.) by using a trap surrounded by septa that contained their synthetic pheromones, i.e., (Z)-11-hexadecenyl acetate for the former species and a 1 : 1 mixture of this acetate and the aldehyde analog for the latter species (Witjaksono, unpublished data). A possible explanation for the incomplete disruption may be that *A. s. cretacea* males were rather strongly attracted to the rubber septa containing epo3,Z6,Z9–19 : H as a disruptant, and males may have the ability to distinguish a lure containing epo3,Z6,Z9–19 : H placed at the center of a trap and those septa separated by 20 cm. Our results suggested that a higher pheromone concentration in a field atmosphere might be necessary for communication disruption of *A. s. cretacea* than for other species.

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CHARACTERIZATION OF ANTIFUNGAL METABOLITES PRODUCED BY *Penicillium* SPECIES ISOLATED FROM SEEDS OF *Picea glehnii*

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Abstract—We screened the microorganisms that are present on the surface of *Picea glehnii* seeds and produced antifungal compounds against *Pythium vexans*, a fungus that causes damping-off. Four isolates of *Penicillium* species that produced patulin, citrinin, palitantin, and arthrographol, respectively, were identified from 149 different microorganisms screened. This study is the first step in an examination of the ecological interaction between host conifers and fungi located on the surface of their seeds.

Key Words—*Picea glehnii*, conifer seedling, root protection, *Pythium vexans*, associated fungi, *Penicillium* spp., patulin, citrinin, palitantin, arthrographol.

INTRODUCTION

Picea glehnii (Fr. Schm.) Masters is one of the most important needle-leafed trees in Hokkaido, Japan, especially in the northern and eastern portions of the serpentine district, in volcanic sand and gravel areas, and in bogs (Tatewaki, 1958). Conifer seedlings are often threatened in the early stages of growth by damping-off fungi, including *Pythium*, *Fusarium* and *Rhizoctonia* species. This disease sometimes also causes a serious problem in tree nurseries (Ito, 1955).

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Once the roots of seedlings are associated with ectomycorrhizal fungi, host plant defenses are enhanced against the fungi that cause damping-off. Many researchers have investigated the defensive mechanisms associated with ectomycorrhizal fungi. The production of antifungal compounds by either the mycosymbiont or the host plant, a barrier effect of the fungal sheath around roots, and nutrient competition between the ectomycorrhizal and pathogenic fungi have all been discussed as possible mechanisms (Zak, 1964; Marx, 1972). A series of experiments that inoculated seedlings with ectomycorrhizal fungi showed that it took a long time to form ectomycorrhizae around the roots of seedlings under nursery conditions. For example, in Kasuya's (1995) experiment, ectomycorrhizae formation was still incomplete 15 months after 2-month-old seedlings of *Pi. glehnii* grown in sterilized soil were inoculated with the ectomycorrhizal fungi, *Pisolitus tinctorius* and *Scleroderma flavidum*. Therefore, it is unlikely that the fungal sheath forms a physical barrier around roots, so protecting young seedlings from pathogenic attacks.

Other defense systems are reported to function in roots inoculated with ectomycorrhizal fungi prior to ectomycorrhiza formation (Stack and Sinclair, 1975; Chakravarty and Unestam, 1987). When sterilized *Pinus resinosa* seedlings growing in test tubes containing a nutrient solution were inoculated with *Paxillus involutus*, followed by an inoculation with *Fusarium oxysporum* 24 hr later, the mortality of the seedlings was significantly reduced, compared with the control, after two weeks (Duchesne and Peterson, 1988; Duchesne et al., 1988). Furthermore, Duchesne et al. (1989) isolated an antifungal compound from a filtrate of the mycosymbiont and identified it as oxalic acid.

It is believed that plants have evolved many chemical defenses against phytopathogens (Ingham, 1973). Therefore, it is possible that several defense systems operate in *Pi. glehnii* seedlings in association with ectomycorrhizae formation, including the production of antifungal compounds by either the mycosymbiont or host plant. We postulated that germinating seeds and seedlings of *Pi. glehnii* might be protected from pathogens in the soil by epiphytic microorganisms that attach to the seed coat and produce antimicrobial compounds. To test this idea, we screened the microorganisms on the surface of seeds to look for those able to produce antimicrobial compounds against the damping-off fungus, *Pythium vexans*. In this paper, we present the results of the screening test. The antifungal compounds produced by the fungi are identified and their roles in the rhizospheric ecosystem are discussed briefly.

METHODS AND MATERIALS

General

Potato–dextrose broth was purchased from Difco and potato–dextrose agar (PDA) medium with 2% agar was prepared in the usual way.

Spectroscopic analyses of the isolated compounds were conducted using the following apparatus. ^{1}H and ^{13}C NMR spectra were recorded on Jeol EX 270 and Bruker AMX500 spectrometers, respectively. 2D NMR (H-H COSY, HMQC, HMBC, and NOESY) to elucidate carbon sequences of the antibiotics were recorded on the Bruker AMX500. The solvent used was $CDCl_3$ and chemical shifts are reported relative to the TMS (^{1}H) and solvent peaks ($\delta = 77.0$ ppm; ^{13}C). EI-MS and EI-HR-MS spectra to determine their molecular weights and formulas were recorded on the Jeol DX 500 spectrometer and FD-MS on a Jeol JMS-SX102A. UV and IR spectra to give information about conjugation systems and functional groups of the compounds were recorded on Hitachi model U-3210 and model 285 spectrometers, respectively. The melting points were determined on a Yanako MP-30 micro-melting point apparatus and are uncorrected. The optical rotations for asymmetric compounds were recorded on a Jasco DIP-370.

Screening Test of Microorganisms Producing Antifungal Compounds

Seeds. Seeds of *Pi. glehnii* were collected at Shirataki, Shiranuka, Oketo, and Teshio in Hokkaido. The seeds were harvested at the first two locations in 1995, from Oketo in 1989, and from Teshio in 1985.

Screening Test. The agar-on-paper method (Shimazu, 1986) was used for the screening. A piece of sterilized filter paper (8.5 cm ID) was put on each agar plate (10 ml of 2% agar in a 9-cm Petri dish) and 2% PDA (10 ml) was layered on top of it. Then, two seeds were put in each plate and incubated at 25°C for 10 days. There were 50 replications per habitat. The filter paper was then transferred to another Petri dish together with the upper layer and stored at room temperature. PDA medium (10 ml) impregnated with mycelial fragments of *Py. vexans* was layered over the remaining lower agar layer, and the plates were incubated at 25°C for 24 hr. When sufficient amounts of antifungal compounds were produced by the fungi and diffused to the lower layer, inhibitory zones appeared in the upper layer containing test mycelia. When this happened, the microorganisms producing the antifungal compounds were isolated from the appropriate colony by the streak culture method.

Identification of Isolated Fungi. Fourteen isolates from a total of 149 examined were selected for further experiments, based on the antifungal potential of the ethyl-acetate-soluble metabolites that they produced against *Py. vexans*. Four of the 14 isolated fungal strains showed remarkable antifungal activity against *Py. vexans*. These fungi were identified by referring to the descriptions of Ramirez (1982); PGS-T5 from the Teshio seeds was identified as *Penicillium cyareum* (Bainier et Sartory) Biourge; PGS-O7 from the Oketo seeds was identified as *Pe. damasceum* Baghdadi; and PGS-S4 and S16 from the Shiranuka seeds were both identified as *Pe. implicatum* Biourge.

Paper Disk Method. The paper disk method was used to evaluate the anti-

fungal activity of the fungal metabolites. The metabolites in each fungal culture were partitioned between ethyl acetate and water. Concentrated ethyl acetate extracts and water layers (each 10 μl) equivalent to 62.5 and 125 μl of each culture filtrate were aseptically charged on paper disks (8 mm ID). Mycelial disks (8 mm ID) of *Py. vexans* freshly grown on 2% PDA were inoculated at the center of plates of the same medium and paper disks, which had previously been dried in vacuo to remove the solvent, were put on the plates 1.5 cm from the edge of the mycelial disks. When the test fungus was *C. herbarum*, plates of 2% PDA impregnated with the spores were used. The plates were incubated at 25°C for 36 hr. The growth inhibition was measured as the width (millimeters) of the inhibitory zone from the edge of the paper disk. Every test was triplicated and the results were averaged.

Fungi. *Pythium vexans* (Oomycetes), a damping-off fungus isolated from the roots of *Pi. glehnii* seedlings in the nursery of Hokkaido University (Kasuya, 1995), was used as a representative test fungus. *Cladosporium herbarum* AHU 9262 (Hyphomycetes) was also used to detect antifungal compounds (Homans and Fuchs, 1970).

TLC Bioautography Assay. This assay was used to determine the activity of compounds soluble in ethyl acetate against *Py. vexans* and *C. herbarum*. Extracts equivalent to 125 and 250 μl of the filtrate were charged on TLC plates (0.25 mm, Kieselgel 60 F_{254}, Merck) and developed in ethyl acetate. The resulting TLC plates were put on PDA plates impregnated with mycelial fragments of *Py. vexans*. The plates were incubated at 25°C for 24 hr. With *C. herbarum*, a spore suspension was sprayed over the developed TLC plates, which were then incubated at 25°C under humid conditions for three days (Homans and Fuchs, 1970). The observed inhibitory zones were correlated with the spots seen on the TLC plates under UV light.

Identification of Antifungal Metabolites Produced by Isolated Fungi

Preparation and Identification of Antifungal Metabolites. Each mycelial disk (8 mm ID) cut from the margin of young colonies of *Pe. cyareum* PGS-T5, *Pe. damasceum* PGS-O7, *Pe. implicatum* PGS-S4, or *Pe. implicatum* S16 grown on PDA, was inoculated into a 300-ml Erlenmeyer flask containing 100 ml of potato dextrose medium and incubated at 25°C for 10 days under stationary culture conditions. Each culture filtrate was extracted with 30 ml of ethyl acetate three times. The combined extracts were washed with saline, dried over Na_2SO_4, and evaporated to give an oily residue, which was diluted to 3 ml with ethyl acetate. The antifungal activity of the ethyl acetate extracts and water-soluble compounds was determined. The ethyl acetate extract from the culture filtrate of *Pe. cyareum* PGS-T5 was charged on TLC plates (0.25 mm, Kieselgel 60 F_{254} Merck) and developed in 50% hexane–ethyl acetate twice to elute

compound **1** (patulin, 3.4 mg from 2.7 ml of culture filtrate, an amorphous powder, R_f = 0.59 in EtOAc). EI-HR-MS determined that the molecular formula of compound **1** was $C_4H_7O_4$ (found 154.0258; calcd. 154.0266). It was identified as patulin on the basis of its ^{1}H and ^{13}C NMR, UV data, and melting point, which were all in full agreement with reported data (Okeke et al., 1993; Paterson and Kemmelmeier, 1990).

The culture filtrates of *Pe. damasceum* PGS-O7 and the two strains of *Pe. implicatum* (PGS-S4 and S16) yielded oily compounds soluble in ethyl acetate. The residuals were crystallized from ethanol, to yield compounds **2** (citrinin, 10 mg from 340 ml of culture filtrate, yellow needles, R_f = 0.10 in EtOAc), and **3** (palitantin, 7.5 mg from 220 ml of culture filtrate, colorless needles, R_f = 0.30 in EtOAc), respectively. Compound **2** from *Pe. damasceum* PGS-O7 has the molecular formula $C_{13}H_{14}O_5$ (EI-HR-MS found 250.0863; calcd. 250.0841) and was identified as citrinin by comparing the spectroscopic results (^{1}H and ^{13}C NMR, UV spectrometry, melting point and $[\alpha]_D$) with reported values (Betina, 1984; Colombo et al., 1980; Paterson and Kemmelmeier, 1990). Compound **3** from *Pe. implicatum* PGS-S4 and S16 has the molecular formula $C_{14}H_{22}O_4$ (EI-HR-MS found 254.1499; calcd. 254.1519) and was identified as palitantin by analyses of its ^{1}H and ^{13}C NMR, UV spectra, melting point, and $[\alpha]_D$, which fully agreed with published data (Mierisova et al., 1996; Hanessian et al., 1989; Paterson and Kemmelmeier, 1990).

The concentrated EtOAc solutes remaining after compound **3** was precipitated from the *Pe. implicatum* PGS-S4 culture filtrate were applied to a silica gel column (Wako gel 60, 19 g) and eluted with ethyl acetate. Fractions 9–13 (2 ml each) containing compound **4** were combined, concentrated further, and subjected to reverse phase column chromatography (26 g of Cosmosil $75C_{18}$-OPN) eluted with MeOH–H_2O (1 : 1). Fractions 28–46 (2 ml each) from the second chromatography step were combined and concentrated to yield compound **4** as an amorphous powder, 2 mg from 760 ml of the culture filtrate, R_f = 0.67 in EtOAc, with the molecular formula $C_{13}H_{14}O_5$ (EI-HR-MS, found 218.0933; calcd. 218.0943). The ^{1}H and ^{13}C NMR, UV spectra, melting point, and $[\alpha]_D$ of compound **4** from *Pe. implicatum* PGS-S4 fully agreed with the published data for arthrographol (Pfefferle et al., 1990).

RESULTS AND DISCUSSION

Of the 149 microorganisms isolated from the surface of *Pi. glehnii* seeds, four showing high antifungal activity against *Py. vexans* were selected by the paper disk method. These four isolated fungi were all identified as *Penicillium* species: *Pe. cyareum* PGS-T5, *Pe. damasceum* PGS-O7, and *Pe. implicatum* PGS-S4 and S16. Ethyl acetate extracts equal to 62.5 and 125 μl of the

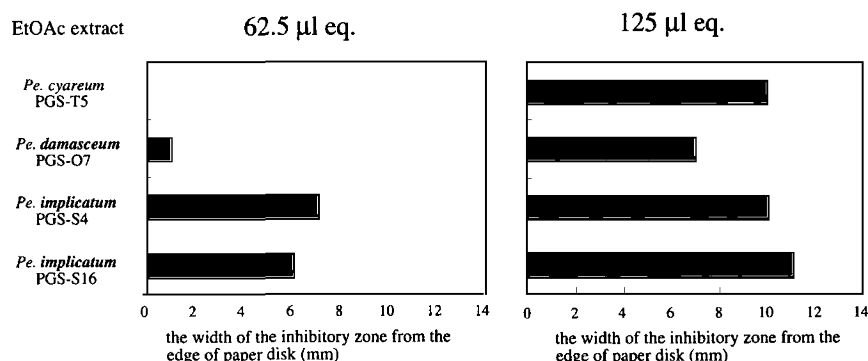


FIG. 1. Antifungal activity of ethyl acetate extracts from culture filtrates of isolated fungi against *Pythium vexans*. The ethyl acetate extracts equal to 62.5 and 125 μl of culture filtrates of *Pe. cyareum* PGS-T5, *Pe. damasceum* PGS-O7, and *Pe. implicatum* PGS-S4 and S16 showed antifungal activity against *Py. vexans*. Ethyl acetate extract equal to 62.5 μl of the culture filtrate of *Pe. cyareum* PGS-T5 did not show antifungal activity, but retardation of mycelial growth around paper disks was observed.

culture filtrates of these strains showed antifungal activity against *Py. vexans* (Figure 1). The activities of *Pe. implicatum* PGS-S4 and S16 were especially remarkable. Although the water-soluble constituents in the culture filtrate of *Pe. cyareum* PGS-T5 also exhibited weak antifungal activity, this activity was due to the incomplete extraction of antifungal components with ethyl acetate (data not shown). The water-soluble metabolites from the other three fungi were inactive.

The antifungal metabolites in the culture filtrates were examined with TLC bioautography using *Py. vexans* and *C. herbarum* as the test fungi. The antifungal zones appearing with each fungus showed essentially the same patterns. Figure 2 shows a typical result with *C. herbarum*. Under guidance of the TLC bioautography, the antifungal compounds in the filtrates were isolated and purified, primarily by chromatographic methods, and were identified as patulin (**1**), citrinin (**2**), palitantin (**3**), and arthrographol (= asperfuran, **4**) by spectroscopic analyses (*see* Methods and Materials). Compound **4** isolated from the filtrate of *Pe. implicatum* PGS-S4 selectively inhibited the growth of *C. herbarum*. The structures of the four antifungal compounds are shown in Figure 3.

Patulin (**1**), the major active compound in *Pe. cyareum* PGS-T5, has been isolated from other *Penicillium* and *Aspergillus* species and is both a potent antibiotic and a carcinogenic and phytotoxic agent (Anslow et al., 1943; Betina, 1984, 1989; Katzman et al., 1944; Nickell and Finlay, 1954; Wang, 1948). Citrinin (**2**) from *Pe. damasceum* PGS-O7 has also been reported in several *Penicillium* and *Aspergillus* species and is a fungitoxic and phytotoxic agent (Betina, 1984, 1989; Robinson and Park, 1966; White and Truelove, 1972; Damodaran

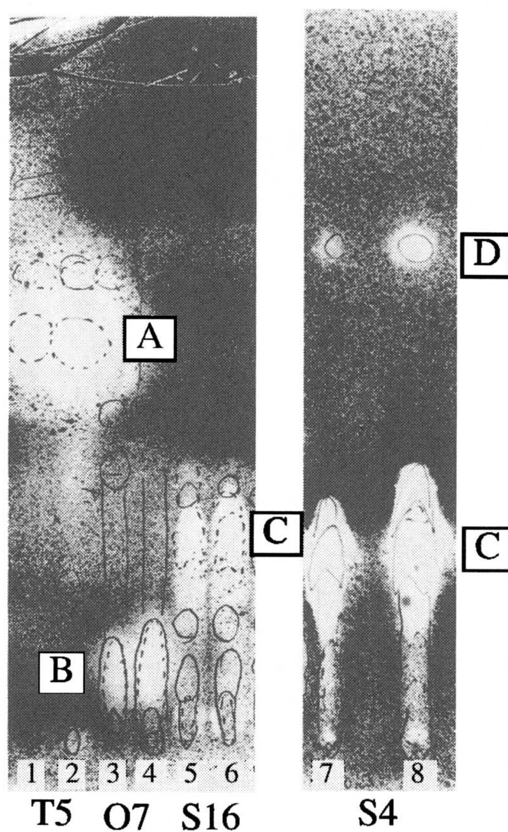
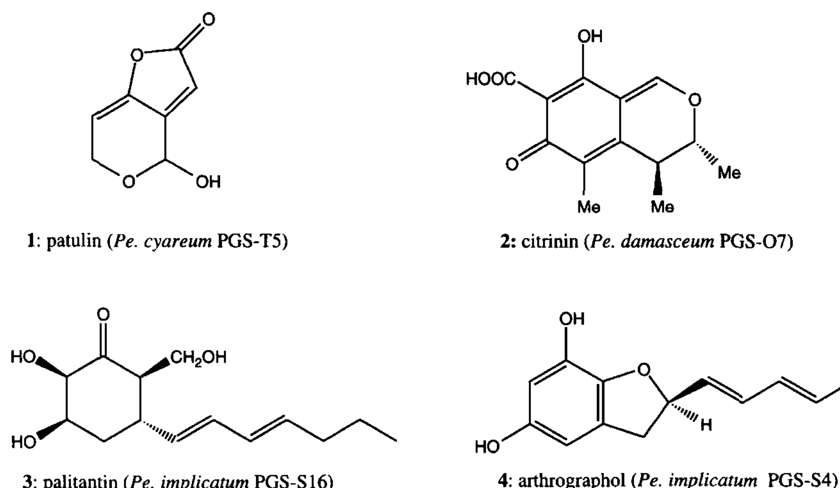


FIG. 2. TLC bioautogram of ethyl acetate solutes from culture filtrates of the positive strains. Ethyl acetate extracts equal to 125 (lanes 1, 3, 5, and 7) and 250 μl (lanes 2, 4, 6, and 8) of culture filtrates on Merck Kieselgel 60 F_{254} plates (0.25 mm) were developed in ethyl acetate, and the chromatograms were then sprayed with a spore suspension of *C. herbarum*. The inhibitory zones labeled with capital letters result from the identified compounds: zone A, patulin (**1**); B, citrinin (**2**); C, palitantin (**3**) and D, arthrographol (**4**).

et al., 1975). These fungitoxins are well-known pollutants of stock foods following fungal contamination. On the other hand, there are no reports of phytotoxic activity by palitantin (**3**), although it is known as an antifungal metabolite in some *Penicillium* species (Birkinshaw and Raistrick, 1936; Curtis et al., 1951; Bracken et al., 1954). Arthrographol is a rare polyketide, which has been isolated as an antifungal metabolite from *Arthrographis pinicola* (Ayer and Nozawa, 1990) and *Aspergillus oryzae* (Pfefferle et al., 1990). There is no description of



1: patulin (*Pe. cyareum* PGS-T5)

2: citrinin (*Pe. damasceum* PGS-O7)

3: palitantin (*Pe. implicatum* PGS-S16)

4: arthrographol (*Pe. implicatum* PGS-S4)

FIG. 3. Structures of isolated antifungal compounds.

its phytotoxic activity. This is the first time that arthrographol has been isolated from the culture filtrate of a *Penicillium* species.

The antifungal activities of the isolated metabolites **1**, **2**, and **3** are shown in Figure 4. Compounds **1** and **2** showed marked antifungal activity against both test fungi when measured in terms of the ID_{50} of mycelial growth. Compound **3** also has relatively high activity against *Py. vexans* (ID_{50} for *Py. vexans*: **1**, ca. 65 nmol; **2**, ca. 80 nmol; **3**, ca. 55 nmol). *Py. vexans* was much more susceptible than *C. herbarum* to these metabolites. However, only the ID_{50} for compound **3** was significantly different when *Py. vexans* and *C. herbarum* were compared (ID_{50} for *C. herbarum*: **1**, ca. 65 nmol; **2**, ca. 92 nmol; **3**, ca. 150 nmol).

The seeds of plants are normally covered with many microorganisms, and germinating seeds encounter more microorganisms that inhabit the soil. During growth, many plants come into contact with particular microorganisms, and this often results in either an association or an infection (Watanabe, 1993). Studying the relationship between *Pi. glehnii* seeds and microorganisms is an important first step in learning about the effects of seed epiphytic microorganisms on seed germination and seedling growth.

All of the microorganisms isolated from the surface of *Pi. glehnii* seeds that produced antifungal compounds were *Penicillium* species. A *Penicillium* species has also been isolated from the surface of *Pi. jezoensis* seeds and seedlings that were unhealthy or blighted (Takahashi, 1980). In this case, the *Penicillium* species seemed to be parasitizing *Pi. jezoensis*. Similarly, *Pe. cyareum* PGS-T5 and *Pe. damasceum* PGS-O7, which were isolated from *Pi. glehnii* seeds, are

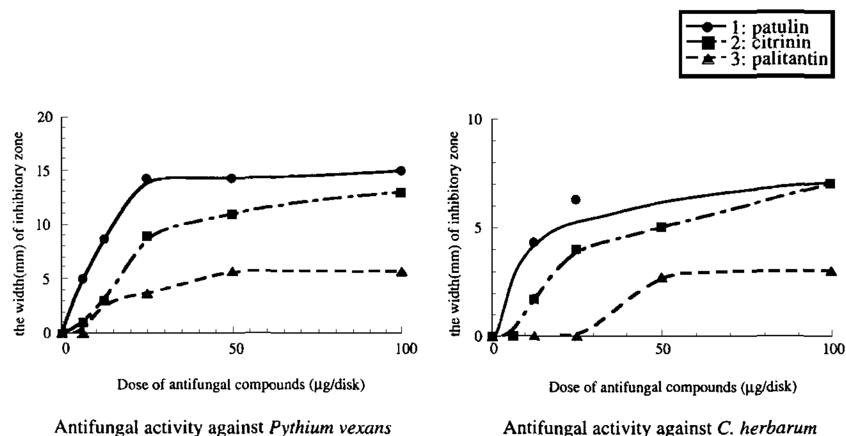


FIG. 4. Antifungal activity of isolated patulin (**1**), citrinin (**2**), and palitantin (**3**). The antifungal activity of each compound was examined using the paper disk method. Five different dosages (6.25, 12.5, 25, 50, 100 μg/disk) of each antifungal compound were tested.

likely to be pathogenic *Penicillium* species, since these strains produced phytotoxins **1** and **2** as the major metabolite, respectively. On the other hand, *Pe. implicatum* PGS-S16 and S4, which produced compound **3**, likely plays a defensive role on the host plant, as **3** does not have phytotoxic activity. A preliminarily examination of the phytotoxicity of all the isolates on lettuce seedlings was made. Further study of these three *Penicillium* species is needed to determine whether they are pathogenic or advantageous microorganisms.

This was the initial search for new interrelationships between tree seedlings and microorganisms and focused on seed epiphytic fungi and seedlings. We found our results encouraging and are conducting further research. *Pi. glehnii* is a model plant for studying the ecological relationships between coniferous trees and seed epiphytic microorganisms.

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PERCEPTION OF OVIPOSITION-DETERRING PHEROMONE BY CABBAGE SEED WEEVIL (*Ceutorhynchus assimilis*)

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Abstract—Following oviposition into a pod of oilseed rape (*Brassica napus*), the female cabbage seed weevil (*Ceutorhynchus assimilis*) marks the pod with oviposition-deterring pheromone (ODP) by brushing it with her eighth abdominal tergite. On an unmarked pod, oviposition site selection was always accompanied by intensive antennation of the pod. Females approaching a freshly ODP-marked pod brought their antennae within 1 mm of the pod but usually did not antennate it before rejecting it for oviposition. Females with the clubs of their antennae amputated continued to discriminate pods from stems or petioles as oviposition sites but showed no behavioral response to ODP. Extracts of volatiles air-entrained from ovipositing weevils failed to inhibit oviposition. Air passed over a behaviorally active extract of ODP did not elicit a detectable electroantennogram response. By contrast, when presented as a gustatory stimulus to the sensilla chaetica of the antennal club, a behaviorally active extract of ODP from postdiapause, gravid females elicited a strong electrophysiological response. This response usually involved more than one cell and displayed a phasic–tonic time course over the recording period of 10 sec. Extract from prediapause (and hence sexually immature) females elicited neither behavioral nor electrophysiological (contact) responses. Thus the ODP of the cabbage seed weevil is sensed primarily by contact chemoreception at the sensilla chaetica of the antennae, and the electrophysiological responses recorded from these gustatory sensilla are of value as the basis of a bioassay to assist identification of the active constituent(s) of the pheromone.

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Key Words—Oviposition-deterring pheromone, host marking pheromone, marker, electrophysiology, contact chemoreception, gustatory sensilla, antenna, behavior, *Ceutorhynchus assimilis*, Coleoptera, Curculionidae, *Brassica napus*.

INTRODUCTION

The cabbage seed weevil [*Ceutorhynchus assimilis* Payk., syn. *Ceutorhynchus obstrictus* (Marsh.), Coleoptera, Curculionidae] is a pest of oilseed rape (*Brassica napus* L.) in Europe and North America. It is univoltine, and the adult becomes sexually mature only after winter diapause (Bonnemaison, 1957; Ankersmit, 1964). In the spring, the female mates and lays her eggs singly into pods where the larvae feed on developing seeds (Alford et al., 1991). The female uses an oviposition-deterring pheromone (ODP) to regulate the distribution of her eggs among pods (Kozłowski et al., 1983; Ferguson and Williams, 1991).

Oviposition-deterring pheromones are well known in other insects (Prokopy et al., 1984). For example, the ODP of the cherry fruit fly (*Rhagoletis cerasi* L.) has been identified and tested for its potential in reducing fruit infestation in a cherry orchard (Aluja and Boller, 1992); it is now undergoing commercial development (Boller and Hurter, 1998). As in other insects (Roitberg and Prokopy, 1987; Roitberg and Mangel, 1988), the seed weevil ODP probably evolved primarily as a marker, enabling an ovipositing female to avoid placing more eggs into a pod than its limited resources can support and thus averting competition between her own offspring. However, this marker also has pheromonal action, inhibiting egg-laying by conspecific females. Egg-laying by seed weevils on caged plots of oilseed rape is markedly reduced at populations of more than 2.5 individuals per plant (Free et al., 1983). This reduction is probably mediated by ODP; if a large proportion of pods is marked with the pheromone, then the energy and time required for a weevil to find an unmarked pod is likely to reduce her individual fecundity (Lerin, 1991). In *Rhagoletis* spp., ODP influences behaviors other than oviposition, modifying the movement of males (Katsoyannos, 1975) and females (Roitberg et al., 1984). To allow for multiple actions, ODPs are often referred to as host marking pheromones (HMP) (e.g., Städler et al., 1994).

The female weevil applies ODP to the pod immediately after oviposition with a brushing action of the eighth abdominal segment. When withdrawn, as at rest, this segment comes into close contact with the invaginated and sclerotized cuticular surface of the seventh urotergite (VII UT) from which ODP can be extracted (Mudd et al., 1997, Ferguson et al., 1999). Washings of glass tubes marked with ODP by weevils and extracts of female VII UT have been tested in a behavioral bioassay developed to assess influence on oviposition (Ferguson and Williams, 1991; Mudd et al., 1997). This bioassay is now being used

to monitor chemical fractions of these extracts for ODP activity in an attempt to purify, identify, and synthesize the pheromone and develop a new integrated control strategy for the pest (Mudd et al., 1997). However, the oviposition bioassay needs large amounts of extract and is time-consuming. Moreover, the seed weevil lays eggs only during a six- to eight-week period of the summer, and it is not easy to culture because it is univoltine and overwinters as a sexually immature adult (Bonnemaison, 1957; Ankersmit, 1964). The development of an electrophysiological bioassay might permit a more efficient use of time and ODP material. Such bioassays were key to the identification of active natural and synthetic host-marking pheromone in the cherry fruit fly, *R. cerasi* (Städler et al., 1994).

Behavioral observations suggest that the decision of a seed weevil to accept or reject a pod for oviposition depends, at least in part, upon cues perceived via antennal sensilla. When selecting a site for oviposition, a female weevil walks back and forth along the pod, antennating the substrate much more intensively than when walking on the stem or petiole (Ferguson and Williams, 1991). Blight et al. (1995) used electrophysiological techniques (electroantennography, EAG, and single cell recording) to investigate olfactory responses of the seed weevil antenna to volatile cues from host plants. Because the ODP is effective in oviposition bioassays for only 1–2 hr (Ferguson and Williams, 1991; Kozłowski, 1991), it may be a volatile substance. However, Kozłowski (1984) failed to demonstrate an EAG response in the seed weevil to air that had been passed over three ODP-marked pods.

No electrophysiological studies have yet been made on contact chemoreceptors in this insect. The antennal club bears three types of sensilla: peglike sensilla and hairlike sensilla, with the ultrastructural appearance of olfactory sensilla, and longer uniporous sensilla chaetica, which appear to be primarily gustatory (contact chemoreceptors) (Isidoro and Solinas, 1992). As the antennal clubs come into close contact with the pod, at least some of the cues used in oviposition site selection (or rejection) are probably sensed via the sensilla chaetica.

The present paper reports investigations into the peripheral detection of ODP by the cabbage seed weevil and the development of an appropriate electrophysiological bioassay.

METHODS AND MATERIALS

Insects

Postdiapause seed weevils for use in bioassays and for making extracts were collected from crops of oilseed rape every two weeks during May, June, and July. They were maintained in mixed-sex groups on cut racemes of oilseed rape (cv. Topas) in a laboratory insectary with a 16L : 8D, 15 : 9°C cycle (Ferguson and

Williams, 1991). Weevils were immobilized by chilling to allow sex determination. Before females were used for behavioral bioassays, electroantennography, or air-entrainment, the reproductive maturity of a sample of insects was checked either by caging them singly for 24 hr with rape pods and dissecting for eggs laid or by observing individuals oviposit.

Prediapause weevils were collected as larvae emerging from cut rape plants in summer and were allowed to pupate in trays of soil maintained in the laboratory insectary (conditions as above) for four to five weeks. Emerging adults were collected and kept on cut racemes of flowering rape. Those to be used for the preparation of prediapause extracts were killed by freezing after 1–14 days. The remainder were kept on flowering rape for five weeks and then transferred to trays (200 × 150 × 50 mm) that contained a layer of moist horticultural compost (75% peat) 30 mm deep. The trays were covered with insect-proof nylon mesh and were maintained at 5°C in the dark for 16–26 weeks to allow weevils to undergo diapause. When weevils were required for the gustatory bioassay before they could be found on the field crop, the trays were brought to room temperature and emerging weevils were placed on cut rape racemes as before.

Video Observations

Oviposition site selection, egg-laying, and pod-marking behaviors were observed with the aid of a color CCD video camera (Panasonic WV-F15E) with attached fiberoptic lights. These were mounted on a motorized *X-Y-Z* table operated remotely by joy-sticks to control motion in three dimensions, enabling insect movement to be tracked accurately in close-up. Six weevils were observed ovipositing into a total of 12 pods and subsequently revisiting each pod two to seven times. Observations were made at 17°C.

Air Entrainment

Volatiles produced by weevil populations confined on oilseed rape racemes (cv. Topas) were collected by a dynamic headspace technique (Blight, 1990). For each air entrainment, 40 cut stems, each bearing about 10 immature pods and a few leaves and flowers, were placed in water-filled conical flasks within a glass vessel (20 liters), and postdiapause, sexually mature gravid female weevils or sexually mature males were introduced. The vessel was maintained in a 16L : 8D light cycle and at 18/15°C (light period/dark period). Purified air (2–3 liters/min) was drawn continuously through the vessel and passed through a tube of Porapak (Q 1.5 g) to collect volatiles.

Weevil behavior within the entrainment vessels was observed for 30 min daily, and the entrainment was discontinued when oviposition became infrequent (after five to six days). The total number of ovipositions and pod markings was

estimated by counting the number of eggs in a sample of 20 pods from each vessel that had contained female weevils. In the first entrainment (experiment 3, Table 1 below), volatiles from a population of 250 females were collected and subsequently eluted from the adsorbent with a mixture of methanol–acetone (1 : 1). In the second entrainment (experiments 4 and 5, Table 1 below), 500 males and 500 females were separately entrained and volatiles were eluted sequentially with pentane and dichloromethane. The extracts were concentrated to a volume of 200 μl by fractional distillation of the solvent in a column of glass helices, followed by evaporation under a gentle stream of nitrogen.

Preparation of Extracts for Bioassay

Air Entrainment Extracts. For presentation in the oviposition bioassay, extracts from air entrainments were prepared in a 7 : 1 mixture of acetone and elution solvent (methanol–acetone, pentane, or dichloromethane) at a concentration of about 40 oviposition equivalents per 10 μl (estimated from egg counts). This solvent mixture is sufficiently polar not to disrupt the waxy cuticle of a pod (Mudd et al., 1997).

Extracts from Glass Tubes. Extracts of ODP for EAG studies were collected from glass tubes (15 mm × 2.2 mm OD) that had been fitted over rape pods (13–16 mm long excluding stigma and style) leaving about 4 mm of the proximal end of the pod exposed. Weevils were allowed to oviposit into the pods and to brush the glass tubes with their abdomen. Immediately after the female had brushed them, the tubes were washed in methanol–acetone (1 : 1). Control extracts were obtained in a similar manner from glass tubes that had been fitted over pods but were not brushed by weevils. The extracts were concentrated under nitrogen to produce solutions containing 20 tube-equivalents per 10 μl.

Extracts from Seventh Urotergite (VII UT). The pheromone was extracted in hexane–ether (1 : 1) directly from VII UT of postdiapause, sexually mature gravid females. Extracts were also made from VII UT of prediapause, sexually immature females (1–14 days old). Dissected VII UT were immersed in a mixture of hexane–ether (1 : 1) and extracted by grinding with a glass rod to disrupt the tissues and by agitation in an ultrasonic bath for several minutes. The cloudy solution obtained was centrifuged at 6000 rpm for 5 min, filtered to remove solid matter, and used to prepare extracts for both oviposition and gustatory bioassays. For the oviposition bioassay, the volume of extract was reduced by evaporation under a stream of nitrogen; it was then dissolved in a sevenfold excess of acetone for application to pods at a rate of four VII UT per 10 μl solvent. For the gustatory bioassay, extracts and solvent controls were evaporated to dryness, redissolved in 20 mM NaCl and presented at a concentration of one VII UT per 10 μl. All extracts were stored at -18°C until use.

Oviposition Behavior Bioassay

The test apparatus and procedure were similar to those described by Ferguson and Williams (1991). Experiments were conducted at 17°C. Oilseed rape stems 50–80 mm long were stripped of all leaves and all but one pod (13–16 mm long excluding style), which was left at the uppermost end. Pods were size-matched and arranged in pairs in a Y or T configuration, with stems placed contiguous to each other and supported in water-saturated porous blocks (Oasis, Smithers Oasis, UK Ltd.). In each replicate of an experiment, a postdiapause female weevil was presented with a fresh pair of pods. Immediately before experiments 1 and 2 (Table 1 below), one pod of each pair (the test pod) was treated by allowing a weevil to oviposit into it and mark it with ODP; the other pod (control) was untreated. When test and control treatments were solvent extracts, 10 μl were applied to pods 10 min before the experiment, allowing time for the solvent to evaporate. At the start of the experiment, the test weevil was placed on the petiole of one of the pods of each pair facing away from the stem. In experiment 2 (Table 1 below), the antennal clubs of the test weevils were removed (by amputation at the distal end of the funiculum) 3 hr before their introduction to the bioassay. Weevil behavior was observed for four or six pod visits, and for each pod the numbers of weevil visits, ovipositions, and pod rejections were recorded. A pod rejection was defined as a visit made to a pod before the visit (if any) when the weevil oviposited into that pod. A minimum of 10 weevils were tested in each experiment. A replicate was accepted only if the weevil oviposited, visited both pods, and completed four or six pod visits within 1 hr.

The numbers of weevils that oviposited first in test or control pods were analyzed for evidence of oviposition deterrence in the test extract by a one-sided exact binomial test with null hypothesis H0: $p \leq 0.5$, where p is the proportion of weevils ovipositing first in the control pod (see Mudd et al., 1997). The null hypothesis, which represents no evidence of test extract activity, was rejected if probability (P) < 0.05. Differences in the mean number of times weevils rejected test and control pods for oviposition were also examined for evidence of activity in the test extract.

Electrophysiology

Electroantennogram (EAG). Recordings from the antennae of postdiapause female weevils were made with silver–silver chloride glass electrodes filled with saline solution [composition as in Maddrell (1969) but without glucose]. The antennae were excised and suspended between the two electrodes. The potential difference (microvolts) between these electrodes was monitored with a high impedance amplifier (Syntech UN-03b) and displayed on an oscilloscope and a chart recorder.

The stimulus (1 sec duration) was delivered into a purified airstream (1

liter/min) flowing continuously over the preparation. The delivery system, which employed a filter paper strip in a disposable Pasteur pipet cartridge, has been described previously (Wadhams, 1990). Test and control samples consisted of washings of 20 glass tubes, brushed and not brushed with ODP, respectively, in 10 μl of solvent (methanol–acetone, 1 : 1). These were applied to filter paper strips and the solvent was allowed to evaporate for 1 min before the strip was placed into the cartridge. Each antenna ($N = 8$) was stimulated in random order with the test sample, the control sample, the solvent only, and with a standard consisting of 10^{-6} g of (Z)-3-hexen-1-ol. Each stimulus was presented once and the peak deflection in potential difference that it elicited (the EAG response) was recorded. EAG responses were analyzed by a split-plot analysis of variance and by comparing the responses to different stimuli with the variance ratio test.

Gustatory Bioassay. Extracts were presented as gustatory stimuli to the sensilla chaetica of the antennal club of postdiapause female weevils. The insects were attached, ventral side up, to a flat plastic stage by means of double-sided sticky tape and immobilized with water-based correction fluid (Tipp-Ex fluid, solvent free; Art no. 4800, Tipp-Ex GmbH and Co. KG, Liederbach, Germany). Glass micropipet electrodes were pulled to give tip diameters of 10–50 μm. The indifferent electrode was filled with 20 mM NaCl and inserted into the rostrum. The recording electrode was filled with a solution of the test material in 20 mM NaCl or with 20 mM NaCl alone (control). In preliminary experiments, the test material consisted of KCl (100 mM), or glucose + sucrose (each 100 mM) or sinigrin (100 mM; a glucosinolate found in the Brassicaceae), but usually it consisted of VII UT extract presented at one weevil equivalent per 10 μl. The recording electrode, connected to the preamplifier by silver wire, was placed over the tip of one sensillum chaeticum. Before every recording from a new sensillum, the tip of the electrode was touched to filter paper to draw off a little liquid and bring new solution to the tip. Electrical activity was recorded for 10 sec after stimulus onset, and a minimum of 60 sec were allowed to elapse between presentation of successive stimuli to the same sensillum. Recorded signals were amplified, digitized, and analyzed with Syntech hardware and software (amplifier Syntech UN-06). Statistical analysis was based on the impulses recorded during the first second after stimulus onset. Data were analyzed at the sensillum level as the signal to noise ratio was low, and usually the responses of single cells were not readily distinguishable.

In the first experiment, responses to VII UT extract from postdiapause, gravid females and responses to the control were compared in 85 sensilla from nine insects (4–17 sensilla per insect). A crossover-type design was used to allow for possible carryover effects of one stimulus upon another. Five of the insects received the control stimulus first, followed by the VII UT extract, and the remainder received the stimuli in reverse order. Data were transformed to natural logarithms [$\log_e(n + 1)$] to allow for skewness and were analyzed by

analysis of variance. In the second experiment, responses to VII UT extract from prediapause females, to VII UT extract from postdiapause, gravid females, and to control were tested in 42 sensilla from four insects (9–12 sensilla per insect). Extracts were presented to each insect in random order because no carryover effect was detected in the first experiment. Each sensillum from the same insect received the stimuli in the same order. Data were analyzed by Restricted Maximum Likelihood (REML) (Searle et al., 1992), which allows for unbalanced data.

RESULTS

Video Observations

Female weevils approaching unmarked pods moved rapidly along the stem and petiole, making little antennal contact with the substrate (Ferguson and Williams, 1991), and passed the nectaries at the junction of the petiole with the pod without hesitation. Females continued along the pod in the same manner for about 5 mm and then slowed their walking pace and started to antennate the pod surface intensively. Following oviposition, the side-to-side abdomen-brushing associated with ODP application was usually most intensive at the proximal end of the pod, close to the nectaries. On two occasions, one individual was observed to apply the tip of her abdomen lightly to the nectaries and the petiole as she walked rapidly away from the pod following oviposition and pod-marking.

Females approached pods freshly marked with ODP in the same way as they approached unmarked pods. However, they usually stopped abruptly when their fore-tarsi had reached the nectaries and their antennae were within 1 mm of the pod; they then turned back from the pod, with neither their tarsi nor antennae having touched it. Sometimes, a female hesitated at the nectaries and walked onto the marked pod, antennating it for a short time in the same way as she would an unmarked pod, and then raised her antennae and walked quickly back to the stem.

Oviposition Behavior Bioassay

Effect of Antennal Club Amputation. Amputation of the antennal club appeared to eliminate the response to ODP in the behavioral bioassay. Intact weevils rejected ODP-marked test pods in 65% of encounters but always oviposited during their first encounter with an untreated control pod, usually ovipositing in it before the test pod (experiment 1, Table 1). By contrast, clubless (amputee) weevils showed little rejection of either test or control pods before ovipositing in them and were as likely to lay their first egg into the ODP-marked pod as

TABLE 1. OVIPOSITION BEHAVIOR BIOASSAY: RESPONSES OF POSTDIAPAUSE FEMALE *Ceutorhynchus assimilis* TO EXPERIMENTAL TREATMENTS

Exp.	Treatment of test pod	Treatment of control pod	Extraction solvent	Weevil equivalents per pod (*N*)	Weevils tested (*N*)	Weevils ovipositing first in control pod (*N*)(=*x*)	P^a	Total pod visits assessed (*N*)	Rejections of (mean ± SE)	
									Test pod	Control pod
1	marked with ODP by a weevil	unmarked	none	1	12	9	0.07	4	1.3 ± 0.28	0 ± 0
2[b]	marked with ODP by a weevil	unmarked	none	1	11[b]	4	0.9	4	0.5 ± 0.25	0.3 ± 0.19
3	extract of air-entrained volatiles from ovipositing female weevils	solvent only	methanol–acetone 1 : 1	ca. 40	22	15	0.07	4	0.7 ± 0.20	0.6 ± 0.16
4	extract of air-entrained volatiles from ovipositing female weevils	extract of air-entrained volatiles from male weevils	pentane	ca. 40	21	11	0.5	4	0.8 ± 0.18	0.8 ± 0.16
5	second extraction of air-entrained volatiles from above ovipositing female weevils	second extraction of air-entrained volatiles from above male weevils	dichloromethane	ca. 40	39	22	0.3	4	0.8 ± 0.14	0.8 ± 0.14
6	extract of VII UT of 1- to 14-day-old prediapause female weevils	solvent only	hexane–ether 1 : 1	4	30	12	0.2	6	0.3 ± 0.15	0.4 ± 0.12
7	extract of VII UT of postdiapause, sexually mature, gravid female weevils	solvent only	hexane–ether 1 : 1	4	10	9	0.01	6	2.8 ± 0.49	0.1 ± 0.10

[a]The probability $P = \text{Prob}\ (X \geq x)$ is based on the binomial distribution with the assumption that under the null hypothesis pods are equally likely to be chosen.
[b]Antennal clubs of test weevils amputated.

into the untreated control (experiment 2, Table 1). Clubless weevils moved with more hesitation on plants than did intact weevils, particularly at the junction of the petiole with the stem, where they appeared to feel their way with their foretarsi. Moreover, unlike intact weevils, they moved the remaining parts of their antennae very little when on pods, holding them in a neutral position. Clubless weevils did not attempt to oviposit on the stem or petiole and, having selected an oviposition site, they performed the normal sequence of oviposition behavior (Kozłowski et al., 1983; Ferguson and Williams, 1991) and marked the pod with ODP.

Response to Air Entrainment Extracts. Weevil behavior within the air entrainment vessels appeared to be normal; they fed on flowers and pods, oviposited, marked pods with ODP, and occasionally flew. Females appeared to cause little disturbance to each other. Egg-laying became less frequent as the pods matured and their condition began to deteriorate due to weevil feeding and oviposition. After five or six days, the entrainments were stopped and >90% of weevils were found to have survived. It was estimated that >2500 ovipositions had taken place in each vessel with female weevils.

Solvents with a range of polarities (methanol–acetone 1 : 1, dichloromethane, or pentane) failed to extract detectable oviposition-deterring activity from Porapak Q after air entrainment with ovipositing females. When these extracts were compared in the behavioral bioassay with controls consisting of solvent only, or with extracts from air entrainment lines containing males, the frequencies with which weevils oviposited first in test and control pods did not differ, and there was no evidence that test pods were rejected more often (experiments 3–5, Table 1).

Influence of Physiological Maturity. When extracts of VII UT from prediapause females (1–14 days old, sexually immature) and from postdiapause females (sexually mature, gravid) were tested in the behavioral bioassay, only the extract from postdiapause weevils was active in deterring oviposition (experiments 6 and 7, Table 1).

Electrophysiology

Electroantennogram. The EAG response elicited by extract from ODP-marked glass tubes (175 ± 26.3 μV) was not different from that elicited by unmarked tubes (199 ± 26.3 μV; $F_{1,21} = 0.41$, $P = 0.53$) but the EAG response to solvent alone was significantly weaker (56 ± 26.3 μV; $F_{1,21} = 16.61$, $P < 0.001$) than responses to either tube extract. The EAG responses to marked tubes, unmarked tubes, and solvent were 68.1%, 77.4%, and 21.8% of the magnitude of the response to the (*Z*)-3-hexen-1-ol standard (257 ± 26.3 μV), which was significantly more active than the other stimuli ($F_{1,21} = 16.61$, $P < 0.001$).

Gustatory Bioassay. The antennal club had 15–18 sensilla chaetica ar-

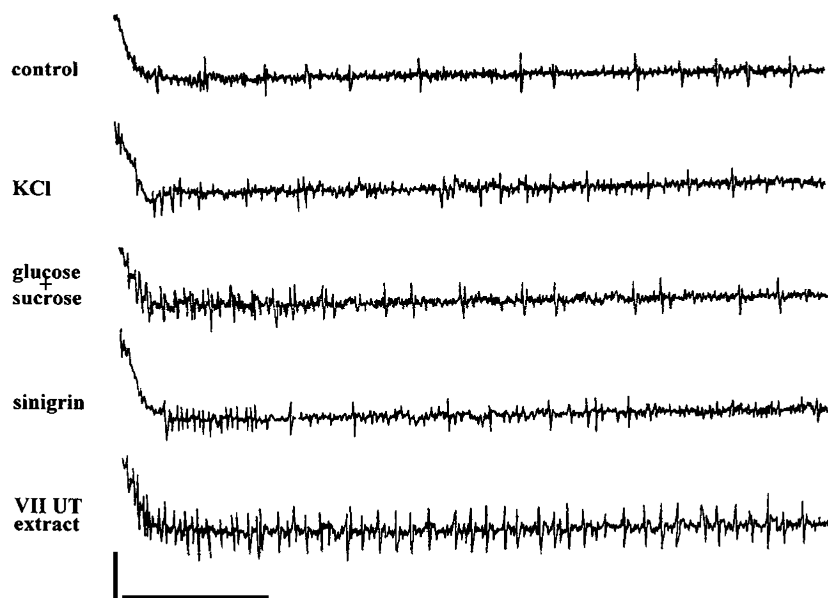

FIG. 1. Examples of electrophysiological responses to various gustatory stimuli recorded from a single sensillum chaeticum of a postdiapause female *Ceutorhynchus assimilis*. Each stimulus was presented in aqueous solution with 20 mM NaCl. Control was 20 mM NaCl only. KCl, glucose, sucrose, and sinigrin were each presented at 100 mM concentration. VII UT extract was from postdiapause, gravid females and was presented at one weevil equivalent per 10 μl. All recordings started 100 msec after stimulus onset. Scale: horizontal bar, 200 msec; vertical bar, 1.0 mV.

ranged in three rings with seven to eight sensilla on the first clavomere, five to six on the second clavomere, and four to five near the tip of the third clavomere. The successful functioning of the experimental preparation was confirmed by the responses obtained from these sensilla to NaCl alone and to NaCl with KCl or with sucrose + glucose or sinigrin (Figure 1). The responses to NaCl and NaCl + KCl were similar, amplitudes of impulses ranging from 0.6 to 1.0 mV. In approximately 12% of all recordings, impulses of two different amplitudes were visible in response to NaCl, indicating the activity of more than one cell. Stimulation with the sugar mixture led to a pronounced increase in impulse numbers, and in a minority of recordings it was possible to distinguish impulses of slightly smaller amplitude than those obtained in response to salts. Forty percent of sensilla tested with sinigrin contained a cell that responded with a burst of impulses in the first second, the impulse amplitudes ranging from 0.4 to 0.7 mV (Figure 1).

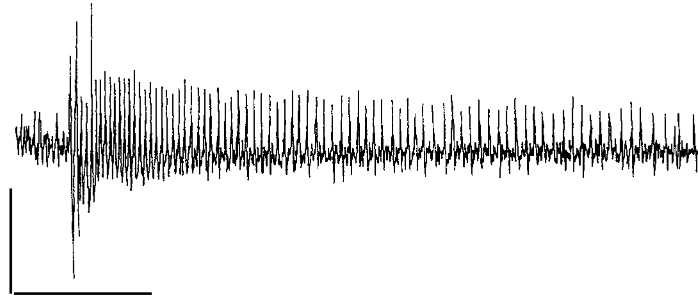

FIG. 2. Example of electrophysiological activity deriving from a single cell in response to VII UT extract. Recording made from a sensillum chaeticum of a postdiapause female *Ceutorhynchus assimilis* during stimulation with extract of VII UT from postdiapause, gravid female *C. assimilis*. Stimulus was presented in 20 mm NaCl. Recording started 100 msec after stimulus onset. The first 80 msec of the presented recording shows electrical noise only. Scale: horizontal bar, 200 msec; vertical bar 0.5 mV.

Extract of VII UT of postdiapause, gravid females usually elicited a strong response. This was usually from more than one cell (Figure 1), but sometimes from a single cell (Figure 2), and it showed a typical phasic–tonic time course. The VII UT extract elicited significantly more impulses in the first second than did NaCl control [means 3.65 and 2.85 (logarithmic scale), respectively, N = 85 sensilla, $F_{1,83}$ = 75.40, $P < 0.001$, SED = 0.092). Extract of VII UT from postdiapause, gravid females elicited more impulses in gustatory sensilla than did extract of VII UT from 1- to 14-day-old, prediapause females or NaCl control (χ^2_2 = 15.3, Wald statistic, $P < 0.001$) (Figure 3).

DISCUSSION

Weevils always approached close to ODP-marked pods before rejecting them for oviposition, and the intense antennation of the pods that they explored is consistent with the use of antennal gustatory sensilla to perceive an ODP of low volatility (Ferguson and Williams, 1991). Amputation of the antennal club, which bears sensilla both of olfactory and gustatory appearance (Isidoro and Solinas, 1992), eliminated the response of female weevils to ODP, providing further evidence that perception of ODP is mediated via chemosensilla on the antennal club. However, the ability of clubless weevils to select pods (rather than stems or petioles) for oviposition remained unimpaired. Cues sufficient to enable pod recognition must be perceived by proprioception, vision, or via nonantennal chemoreceptors (e.g., sensilla on the maxillary or labial palps or on the tarsi). The delay between the arrival of an intact weevil on a pod and the start of anten-

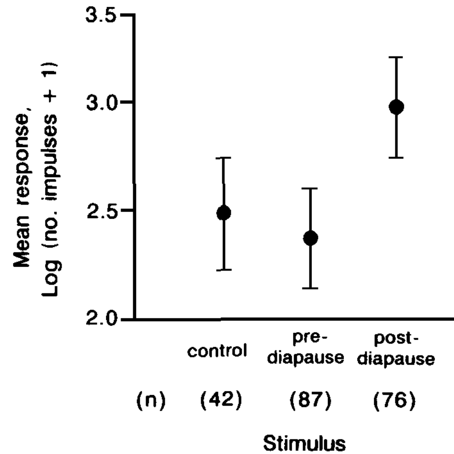

FIG. 3. Comparison of responses to VII UT extracts from pre- and postdiapause female *Ceutorhynchus assimilis* recorded from sensilla chaetica of postdiapause female *C. assimilis*. *Y* axis: mean numbers of impulses recorded in the first second of stimulus presentation after $\log_e(n + 1)$ transformation (on this scale 2.0 and 3.0 represent 6.4 and 19.1 impulses, respectively); bars indicate ±1 SE. Each stimulus was presented in 20 mM NaCl. The stimuli were: control, 20 mM NaCl only; VII UT prediapause, extract of VII UT from 1- to 14-day-old prediapause females; VII UT postdiapause, extract of VII UT from postdiapause, gravid females.

nation of the substrate is consistent with this, antennal activity being initiated only in response to pod recognition. Tarsal contact chemoreceptors are known to exist in other insects (Städler, 1976; Mitchell and Harrison, 1985) and appear to play an important role in oviposition site selection in *Delia radicum*, another pest of *Brassica* spp. (Roessingh et al., 1997). In the cherry fruit fly they mediate the perception of ODP (or HMP; Städler et al., 1994).

Weevils usually were able to reject a freshly marked pod on reaching its nectaries, without touching it. Therefore, ODP must have sufficient vapor pressure to be detected over a distance of <1 mm from the pod without direct contact. Detection might be mediated either by olfactory sensilla or by contact chemoreceptors acting in an olfactory manner over a short distance (Dethier, 1972; Städler and Hanson, 1975). The most intensive application of ODP to pods took place close to the petiole. Concentration of ODP application at the proximal end of the pod may assist weevils to recognize quickly that it is marked, conferring a time-saving benefit in the assessment of pods for oviposition. This benefit only accrues to the female that applied the ODP if she is likely herself to revisit the pod, i.e., if the ODP regulates her own behavior as well as acting as a pheromone. A small amount of ODP may sometimes be deposited on the petiole by abdomen-brushing following oviposition, and this could influence

pod rejection. However, brushing of the petiole was observed only occasionally and was slight in comparison to pod brushing. Moreover, insects approaching a pod rarely touched the petiole with their antennae, and they never rejected a pod until they had reached the nectaries. The function of abdomen-brushing on the petiole is not clear; perhaps it serves to remove cuticular material that was collected on the abdomen from the pod.

Failure to collect behaviorally active material by air entrainment of ovipositing weevils also suggests that ODP may not be very volatile. Indeed, ODP has been shown to be sufficiently involatile to be recovered from an aqueous extract by freeze-drying (Mudd et al., 1997). Low ODP volatility could be of adaptive significance if it confines the deterrent signal to a specific pod, allowing oviposition by weevils in neighboring unmarked pods.

The absence of a detectable EAG response to ODP does not preclude a role for olfaction in ODP perception, even though identical test extracts made at the same time gave strong responses in the oviposition behavior bioassay (Mudd et al., 1997). If the number of olfactory cells responding to ODP are few, then it may not be possible to detect their signals against the electrical activity averaged across the whole antenna. The significant EAG activity elicited by extracts both of ODP-marked and unmarked tubes probably arose from plant volatiles collected through contact of the tubes with pods.

The electrophysiological response elicited in antennal sensilla chaetica by an aqueous solution of VII UT extract from postdiapause, gravid females contrasted with the lack of response to a similar extract from 1- to 14-day-old, prediapause females. Consistent with this, when the same extracts were tested in the behavioral bioassay, weevils were deterred from oviposition by the postdiapause VII UT extract but not by the prediapause VII UT extract. This is strong evidence for a relationship between electrophysiological activity and behavioral activity.

Our evidence is consistent with the conclusion that the ODP of the cabbage seed weevil is sensed primarily by contact chemoreception by sensilla chaetica on the antenna. We have preliminary evidence that responses to ODP can be recorded from the sensilla not only of postdiapause females but also of prediapause females (Ziesmann, personal communication). This would enable the gustatory bioassay to be used for a more extended period each year than is possible with the oviposition behavior bioassay. Furthermore, it is hoped that characterization of responses to VII UT extract by individual cells of the sensilla chaetica will refine the discriminatory power of the gustatory bioassay. The oviposition behavior and gustatory bioassays have been used recently in combination with histological studies to establish the site of production of ODP and to relate weevil age to ODP production in more detail than presented here (Ferguson et al., 1999). At present, both bioassays are being used in tandem to test chromatographic fractions of VII UT extract in order to identify the active constituent(s) of ODP.

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REPRODUCTION AND URINE MARKING IN LABORATORY GROUPS OF FEMALE COMMON VOLES *Microtus arvalis*

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Abstract—In laboratory groups of common voles, *Microtus arvalis*, consisting of one mother, two of her daughters and one male, mainly the mother reproduced. The three females spent most of the time in close body contact. By using a modified method of coloring the urine of the three females by nonmetabolizable, nontoxic dyes, we found that mothers display a higher urine marking intensity than their daughters, and urine marks from the mother were distributed more frequently than those from the daughters. The results suggest that both pheromonal and behavioral mechanisms are involved in the regulation of reproduction in matriarchal groups of common voles.

KEY WORDS—Reproduction, urine marking, nonmetabolizable dyes, spacing behavior, olfactory communication, pheromones, social interactions, *Microtus arvalis*, Rodentia.

INTRODUCTION

Common voles (*Microtus arvalis*) undergo very high annual and multiannual population fluctuations (Frank, 1956a; Heise and Stubbe, 1987) and, in some years, they become a major agricultural pest. Their extremely high fecundity (Heise et al., 1991) results, in part, from their social organization. During the breeding season (usually from March to September/October), most breeding females live in territorial groups of two to six in large common burrows, breed synchronously (Frank, 1956b; Boyce and Boyce, 1988), and probably raise their young communally (Rozenfeld, unpublished). Data from field and laboratory

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studies suggest that females in territorial groups are genetically related (Blumenberg, 1986; De Jonge, 1983; Dobly, unpublished). Young females may be precociously sexually mature at an age of 17–19 days, but usually reach their sexual maturation at a mean age of 27.4 days (Frank, 1957; Boyce and Boyce, 1988; Tkadlec and Zejda, 1995). At high density, early cessation of female reproductive activity was observed in local populations (Wieland et al., 1989).

It is well known in various rodent species that reproduction of adult females as well as puberty of the young of both sexes can be modulated by urinary chemosignals (Vandenbergh, 1986; Brown, 1987; Werner and Clarke, 1990). In some rodent species [e.g., *Mus musculus* (Brown, 1987), *Peromyscus maniculatus* (Terman, 1984), *Microtus pinetorum* (Boyer et al., 1988)], pheromones that inhibit reproduction have been found in the urine of grouped females, leading either to spontaneous pseudopregnancy or anestrus in another sexually mature female or to a delay in the onset of puberty in young females. This regulating mechanism is probably not a laboratory artifact since it was observed in natural and seminatural populations of house mice (Massey and Vandenbergh, 1980) and in some vole species [*Clethrionomys glareolus* (Bujalska, 1970), *C. rutilis* (Gilbert et al., 1986), *C. rufocanus*, (Kawata, 1987)].

As in bank voles (Rozenfeld and Denoël, 1994), breeding female common voles mark their territories with urine and the intensity of urine marking correlates with the social rank of a female within a breeding group (Heise, 1999). We, therefore, hypothesize that urinary pheromones could be involved in the regulation of reproduction in crowded groups of genetically related female common voles by establishing a "reproductive dominance" in mothers in matriarchal groups.

As male common voles visit territorial female groups only occasionally (Frank, 1956b), and presumably stimulate estrus in all females of the group [*M. arvalis* are induced ovulators (Breed, 1972)] by an inducing chemosignal in their urine, a permanent contact between young females and their mother's urinary inhibiting pheromones would be necessary to override the effect of stimulating pheromones from males. This hypothesis is based on studies in house mice showing that the efficiency of reproduction inhibiting pheromones emitted from females depends on frequent urine deposition and/or renewal by the emitter to ensure permanent contact between the recipient and the pheromone (Drickamer, 1983).

For laboratory breeding groups of female common voles we expected that mothers only will reproduce and that mothers will intensively mark their territory to ensure a permanent distribution of urinary pheromones that may regulate the reproductive activity of their daughters within their territory.

METHODS AND MATERIALS

Animals and Rearing Conditions. All animals were first- or second-generation descendants of wild *Microtus arvalis* caught in the southern part of Belgium.

They were kept in an outbreeding system in a rearing room at 20–22°C and in a reversed light cycle (16L:8D) prior to the beginning of the experiment. Seven groups of three females (mother and two daughters) were established as preexperimental groups and kept in polycarbonate cages (22 × 34 × 15.5 cm) until the beginning of the experiment.

In six of the seven groups, mothers were 3.5–4 months old and their daughters were 5–6 weeks old. The seventh group consisted of a 7-month-old mother and two of her 3.5-month-old daughters.

All mothers were in nonbreeding condition. The daughters of each group were virgins and presumed to be sexually mature because young female common voles usually reach sexual maturity at a mean age of 27.4 days (Frank, 1957; Tkadlec and Zejda, 1995).

Stud males came from the same breeding stock and were 3–5 months old. They were all sexually experienced.

Experimental Conditions. All groups were transferred to the experimental room (under the same environmental conditions) 10 days prior to the beginning of the experiment. For the experiment, each group was kept in a rectangular aluminum enclosure, measuring 106 × 55 × 49 cm, equipped with two aluminum nest boxes measuring 15 × 15 × 8 cm with two holes as exits (4.5 cm diam.), and one polyvinyl chloride tunnel (33 cm long × 6.5 cm wide). The animals in the enclosure were provided with water and food (hamster food U.A.R. "105") ad libitum, supplied by one water bottle and one rack (Figure 1). The animals remained in the enclosure for seven days.

On the first day of the experiment, females were weighed, individually marked by fur clipping, and placed in the enclosures with an unrelated, sexually experienced male. Females were weighed again on days 3, 5, and 7 of the experiment in order to include differences in their weight in the analysis of their urine marking intensity.

On days 2, 4, and 6 each female received a 0.5-ml injection of a 0.2% solution of a nonmetabolizable, nontoxic dye in physiological solution of NaCl subcutaneously into the neck region in order to color the urine. We used fluorescein sodium (Merck), rhodamine B, and methylene blue as dyes. The choice of the dyes on successive injection days was random, but was the same for each group.

Prior to injections, the floor of each enclosure was lined with cardboard in order to make colored urine marks visible. Preliminary experiments showed that urine was colored within 12–16 hrs following injection. After 24 hrs, the cardboard was removed from the enclosure after the position of all items (nest boxes, rack, etc.) had been marked on it.

On day 7, the animals were removed from their enclosures. The male was isolated, and the females of each group were kept together in a small cage (22 × 34 × 15.5 cm) until they had litters. For each group, any female that had a litter (mother or daughters) and the number of pups were recorded.

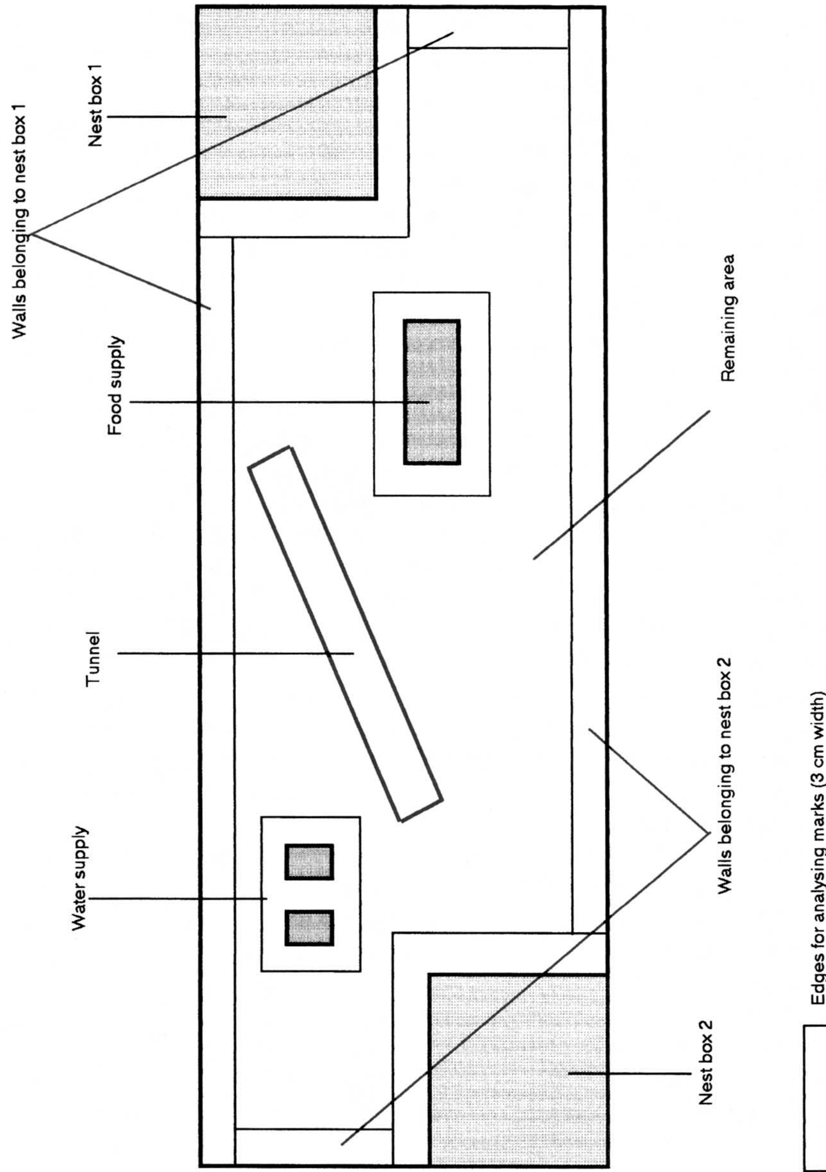

FIG. 1. Diagram of the experimental enclosure.

Analysis of Urine Marks. For analyzing urine marks, we drew 3-cm-wide edges around the nest boxes, the food supply, the water supply, and along the walls of the enclosure (Figure 1). Within these edges, as well as on the remaining area, we counted all colored urine marks and divided them into three categories according to their shape: (1) stripes—long narrow lines of urine (less than 0.3 mm in width), (2) spots—round urine depositions up to a size of 0.4 mm in diameter, and (3) other marks—any other forms of urine deposition.

The number of urine marks was counted for each dye, each shape of urine mark (category), each experimental group, and each injection day separately.

To compare the intensity of urine marks around the different items, we calculated the area of each mark by measuring the length and width of a stripe (which was regarded as an ellipse) and the diameter of a spot (which was regarded as a circle). The areas of all other marks were measured by using an overlay of transparent metric paper.

We estimated the relative marked area around each item as follows: The sum of the marked areas within the edges for analyzing marks of each item was related to the entire possible marking area around this item and expressed as a percentage marked area of this item.

We classified the following items in the enclosure:

Np	preferred nest box (where the animals were seen most often)
N	the other nest box
W	water supply
F	food supply
T	tunnel
Wp	walls near the preferred nest box Np
W	walls near the other nest box N
Rem	remaining area

The classification of the two nest boxes (preferred and other) was based on the frequency with which the animals were seen in each of the two nest boxes during a seven-day observation period with three observations per day (see below).

Behavior Analysis. Three times per day the place at which each animal of the group was seen in the enclosure and its physical contact with other members of the group were recorded. From days 1 to 7, these three recordings were conducted for all groups between 09:00 and 09:30 hr, between 12:30 and 13:00 hr, and between 17:30 and 18:00 hr, shortly before the dark period.

The associations between the animals were classified as follows:

fffm	all four animals in physical contact
fff-m	all females together and the male alone
fm-ff	one female with the male while the other two females stayed together
ffm-f	two females with the male, one female alone
f-ff-m	one female alone, two females together, male alone
f-f-f-m	all four animals separated

Data Analysis. The relative frequency of each association between the animals over the seven experimental days was calculated for each group. Significant differences in the mean occurrence of observed associations over all groups were analyzed by using the one-way ANOVA of Meddis (1984).

The mean number of differently colored urine mark categories (according to the above described shapes) was compared for each injection day by using the Meddis one-way ANOVA with the specific prediction that adult females (mothers) mark more frequently than their daughters. The mean percentages of the marked area around the different items of the enclosure were also compared by using the one-way ANOVA of Meddis.

The two-tailed Wilcoxon's matched paired test was used to compare the percentage of marked area produced by the mother with the average of those produced by the two daughters for each item separately.

RESULTS

Reproductive Success in Grouped Females. Reproduction occurred in six of seven groups. In five groups, only the mother reproduced. In one group in which daughters were older than in the other experimental groups, the mother and one of her daughters reproduced. The mean litter size was 3.5 (range 2–5).

Associations Between Members of Groups. The mean relative frequency of the occurrence of different associations between animals is illustrated in Figure 2. In 70.1% of all observations, the three females stayed together, either with the male (fffm, 36.5%) or without him (fff-m, 34.1%). Both associations were more frequent than the four other types of associations (MANOVA, $H = 27.19$; $P < 0.05$). In 41% of all observations the male was seen on his own (fff-m, f-ff-m) and in 23% either together with one or with two females. In all cases, when a male was seen in close body contact with one female (fm-ff, Figure 2) this female was often one of the daughers (=73% of those cases).

Urine Marking Behavior. All females scent marked their home area with urine. The mean number of scent marks left in the enclosure by each female of

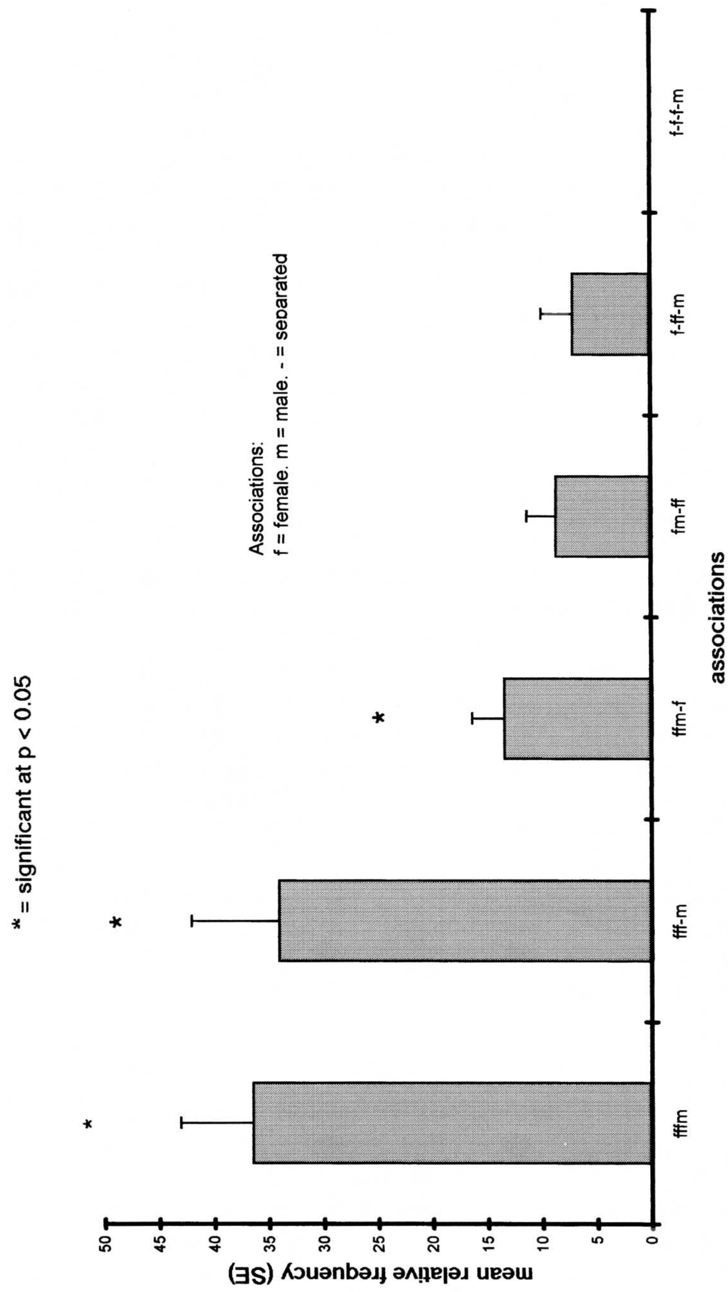

FIG. 2. Mean relative frequency of different associations in groups of one mother, two of her daughters, and one male.

a group is shown in Figure 3. At 24 hr following the first dye injection in which mothers were injected with rhodamine and their daughters with either methylene blue or fluorescein, urine marks of the mothers were more frequent than those emitted by their two daughters (MANOVA, $z = 3.24$, $P < 0.001$). During the second injection day, when all mothers received an injection of methylene blue, differences in the number of marks were not significant. During the third injection day, when all mothers received an injection of fluorescein, yellow-colored urine marks were more frequently deposited than red and blue ones (MANOVA, $z = 2.03$; $P < 0.05$).

All animals received the same amount of colored solution of NaCl. Differences in the weight between the animals might cause a dilution of the urine marks as heavier animals drink more water. This could lead to the excretion of less visible marks that may affect the data analysis. We therefore corrected the number of marks deposited by the mother by a factor derived from the different weights of mothers and daughters per group. However, the same results were obtained (on injection day 1, MANOVA, $z = 3.28$; $P < 0.001$, on injection day 3, MANOVA, $Z = 2.07$, $P < 0.05$).

Considering the three categories of urine depositions separately (spots, stripes, other marks) differences in the number of urine marks between mothers and their daughters were confirmed (Spots: on injection day 1, MANOVA, $z = 3.42$, $P < 0.001$, and on injection day 3, MANOVA, $z = 2.07$, $P < 0.05$; stripes: on injection day 1, MANOVA, $z = 3.41$, $P < 0.001$, and on injection day 3, MANOVA, $z = 1.71$, $P < 0.05$; others: on injection day 1, MANOVA, $z = 2.81$, $P < 0.05$, and on injection day 3, MANOVA, $z = 1.67$, $P < 0.05$).

Figure 4 shows the results of the comparison of the percentage of marked area between the different items of the enclosure. Marks were mainly deposited around the preferred nest box and in the tunnel (MANOVA, $H = 37.32$, $P < 0.001$).

The comparison of the percentage of marked area, produced by the mothers, with the average of those produced by the two daughters for each item, showed that mothers marked the remaining area more intensively than their daughters (Wilcoxon's matched paired test, $z = -1.9645$, $P < 0.05$) (Figure 5).

DISCUSSION

The present study shows that in matriarchal groups of female common voles, consisting of one mother and two of her daughters, all females contribute to the scent marking of their home area. This communal scent marking probably plays an important role in group cohesion. Mothers, however, scent mark at a higher rate than their daughters and do so preferentially in areas that are regularly visited by their daughters.

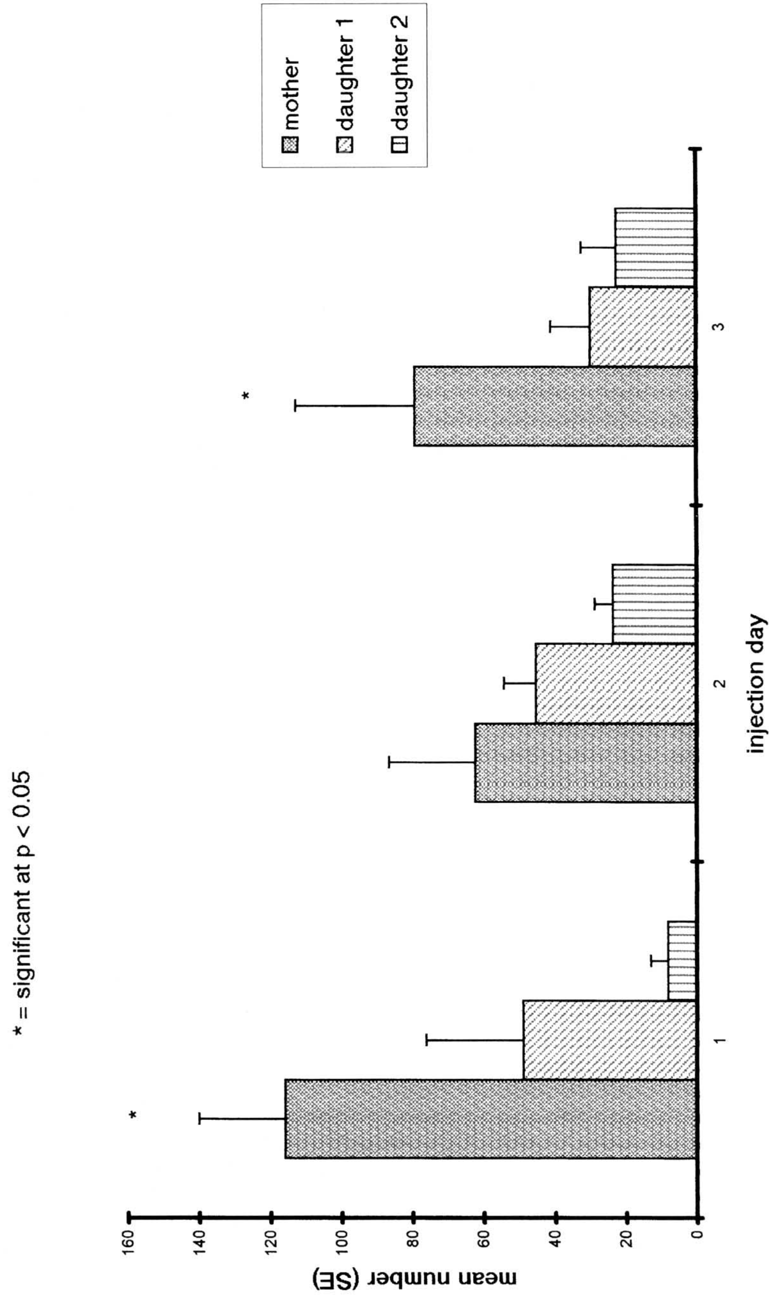

FIG. 3. Mean number of colored urine marks left by three females in groups of one mother, two of her daughters, and one male on three successive days.

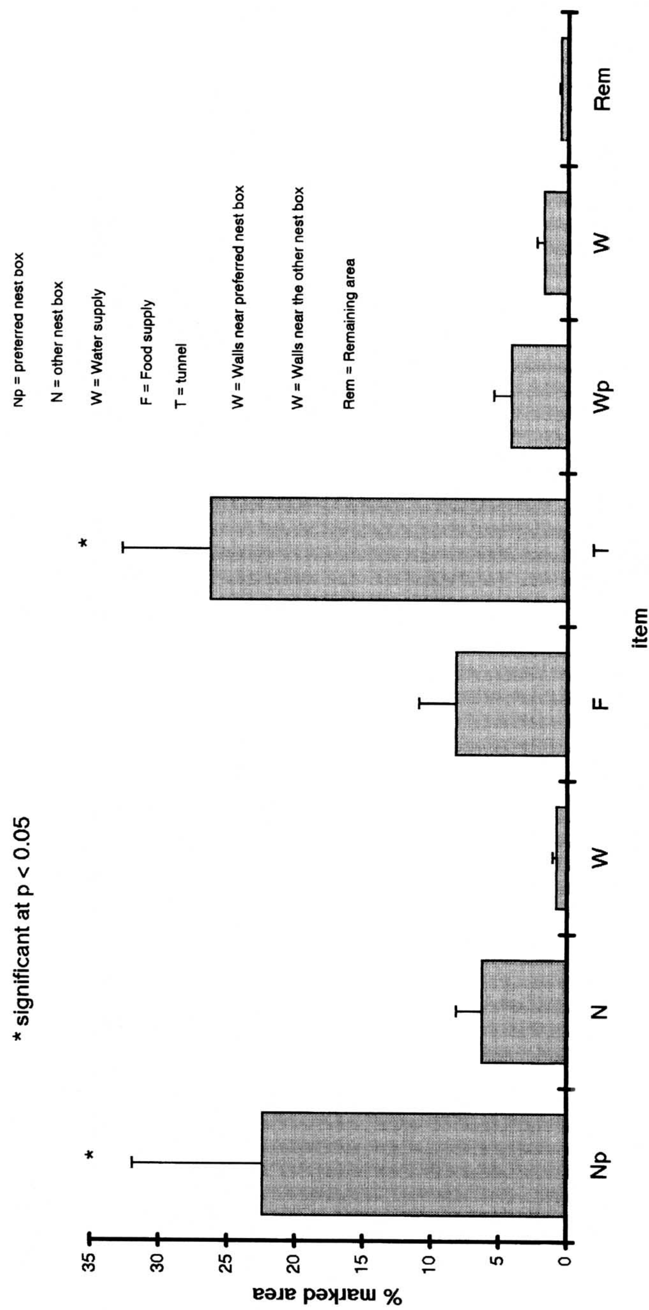

FIG. 4. Comparison of the percentage of marked area around different items in the experimental enclosures.

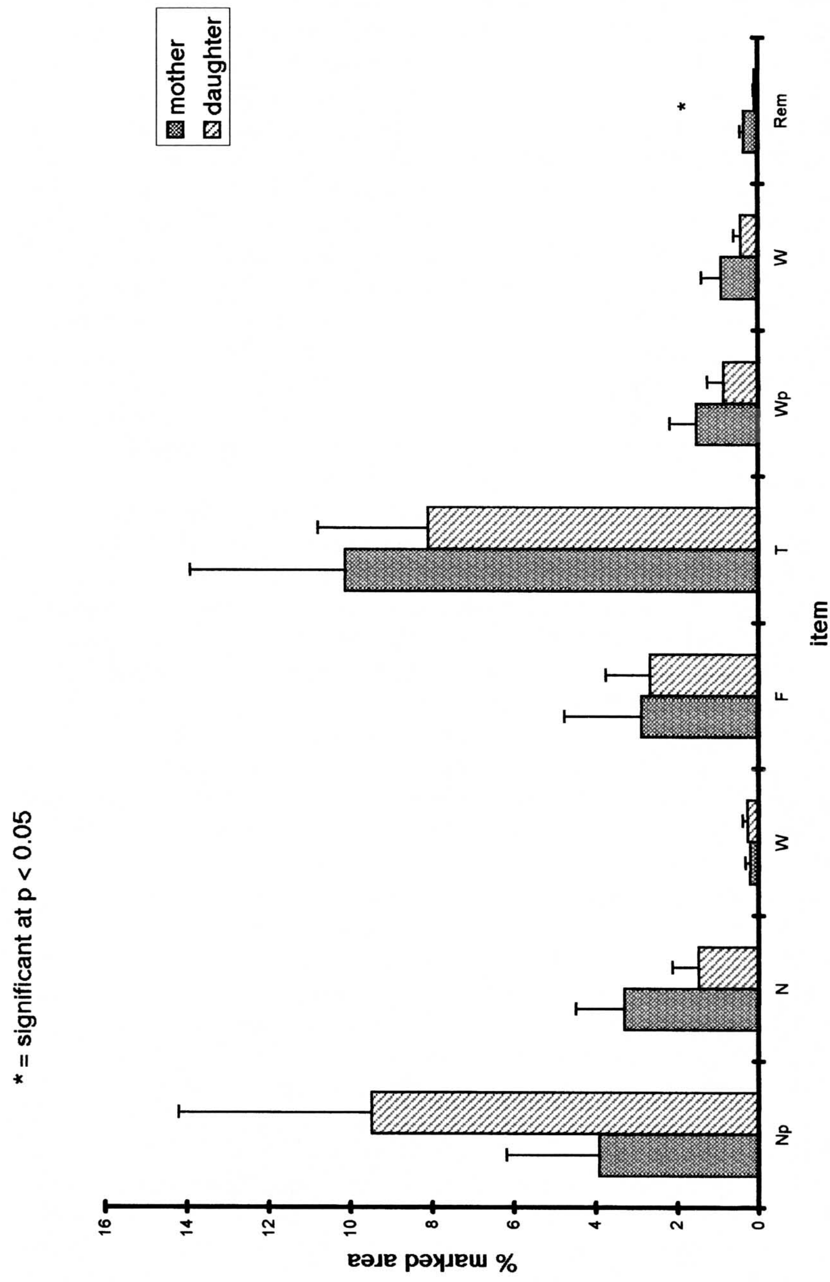

FIG. 5. Comparison of the percentage of marked area produced by mothers and by daughters per item of the experimental enclosures.

Our experiment, designed to mimic the situation of a very high population density with no possibility of emigration, suggests that, at high densities, mothers may reduce the reproductive activity of their daughters. All daughters in our experimental groups were presumed to be sexually mature (older than 27.4 days, which is the mean age of sexual maturation of young female common voles) (Frank, 1957; Tkadlec and Zejda, 1995), but most of them did not reproduce. However, it is known that sexual maturation and growth of young female common voles can be delayed due to high population densities (Heise et al., 1991, 1992). The results of our study may therefore indicate that in our experimental groups the reproduction of young females was either inhibited due to their delayed sexual maturation or the inhibition of physiological processes leading to successful reproduction (e.g., suppression of ovulation, failed implantation etc.). The reproduction of one daughter in the group with the oldest daughters, on the other hand, suggests that the reproductive inactivity of the young females was due to a delay in their sexual maturity. However, this has to be proved in further experiments.

Inhibition of sexual maturation/reproduction in young female rodents is well known to be a density-dependent phenomenon (Vandenbergh and Coppola, 1986; Massey and Vandenbergh, 1980; Skryja, 1978). Pheromones, emitted in the urine of rodents, contribute to the regulation of reproduction by intraspecific interactions (Vandenbergh, 1986). Because they are often volatile (Jemiolo et al., 1989) and fade with time (Sipos et al., 1993), their effect must be maintained by a frequent renewal of urine marks (Wolff and Powell, 1984; Ferkin et al., 1997) or by a permanent contact between the pheromone emitter and the recipient. In our experiments, intensive scent marking at privileged sites by mothers and the permanent physical contact between mothers and their daughters suggest that both pheromonal and behavioral mechanisms are involved in the regulation of reproduction in matriarchal groups of common voles.

We did not test the effect of the mothers' urine by itself on the sexual maturation/reproduction of young female common voles. However, Brant et al. (1998), showed that a reduction in the litter production of young female pine voles (*Microtus pinetorum*) is mainly regulated by behavioral interactions between daughters and their parents rather than by the effect of chemical cues.

The higher intensity of urine marking by the mothers simply may be age-dependent in comparison to that of their daughters. On the other hand, studies showed that scent marking correlates with the social status of an animal (Rozenfeld et al., 1987; Gosling, 1990) and affects its reproductive activity (Gerlach, 1996; Heise, 1999). Mothers in our experimental groups, therefore, may advertise their reproductive dominance in their marked territory to other females and regulate the fertility of daughters that are forced to remain in the communal burrow when no emigration is possible.

The establishment of territorial matriarchal groups in *M. arvalis* is con-

sidered to contribute to an efficient distribution of urine pheromones between the females. In species where females live solitarily and female–female interactions are restricted to mother–daughter relationships or occasional encounters between unrelated females (Bujalska, 1985; Rozenfeld and Denoël, 1994), the role of pheromones may be less important for population regulation than other parameters (e.g., vacant territories) (Ylönen et al., 1988).

The results presented were obtained by coloring the urine of the tested animals by injection of nontoxic, nonmetabolizable vital dyes (New, 1958; Frantz, 1972). In previous studies dyes were administered orally with food (Rozenfeld et al., 1987) or drinking water (Heise, 1999), or they were incorporated into beeswax pellets implanted subcutaneously (*Microtus montanus*, Jannett, 1981) so that feces, urine, and/or internal structures become marked. Both methods have logistic problems. Animals must either be isolated temporarily from their social group until they have ingested a sufficient quantity of dye, or they have to be anesthetized. The method of injecting solutions of physiological serum and dyes into the animals reduces stress by eliminating the need for both anesthetization and a long isolation of the animals from their social group.

We used methylene blue, rhodamine B, and fluorescein as dyes, but we could not detect significant differences between the number of urine marks set by the mother and those set by the two daughters when the mother received methylene blue. Methylene blue seems to have a higher fade-out rate than the other two dyes. The use of nonmetabolizable dyes allowed us to follow the marking behavior of each female in matriarchal groups and to obtain some indirect evidence that pheromonal based reproduction inhibiting mechanisms may exist in groups of related female common voles. We consider the described method promising as an approach to obtain more detailed information on the role of urine marking in social interactions of rodents.

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EFFECTS OF LIGHT AND NUTRIENT AVAILABILITY ON ASPEN: GROWTH, PHYTOCHEMISTRY, AND INSECT PERFORMANCE

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Abstract—This study explored the effect of resource availability on plant phytochemical composition within the framework of carbon–nutrient balance (CNB) theory. We grew quaking aspen (*Populus tremuloides*) under two levels of light and three levels of nutrient availability and measured photosynthesis, productivity, and foliar chemistry [water, total nonstructural carbohydrates (TNC), condensed tannins, and phenolic glycosides]. Gypsy moths (*Lymantria dispar*) and forest tent caterpillars (*Malacosoma disstria*) were reared on foliage from each of the treatments to determine effects on insect performance. Photosynthetic rates increased under high light, but were not influenced by nutrient availability. Tree growth increased in response to both the direct and interactive effects of light and nutrient availability. Increasing light reduced foliar nitrogen, while increasing nutrient availability increased foliar nitrogen. TNC levels were elevated under high light conditions, but were not influenced by nutrient availability. Starch and condensed tannins responded to changes in resource availability in a manner consistent with CNB theory; levels were highest under conditions where tree growth was limited more than photosynthesis (i.e., high light–low nutrient availability). Concentrations of phenolic glycosides, however, were only moderately influenced by resource availability. In general, insect performance varied relatively little among treatments. Both species performed most poorly on the high light–low nutrient availability treatment. Because phenolic glycosides are the primary factor determining aspen quality for these insects, and because levels of these compounds were minimally affected by the treatments, the limited response of the insects was not surprising. Thus, the ability of CNB theory to

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accurately predict allocation to defense compounds depends on the response of specific allelochemicals to changes in resource availability. Moreover, whether allelochemicals serve to defend the plant depends on the response of insects to specific allelochemicals. Finally, in contrast to predictions of CNB theory, we found substantial allocation to storage and defense compounds under conditions in which growth was carbon-limited (e.g., low light), suggesting a cost to defense in terms of reduced growth.

Key Words—Carbon–nutrient balance theory, forest tent caterpillar, growth–differentiation balance theory, gypsy moth, *Lymantria dispar*, *Malacosoma disstria*, phenolic glycosides, *Populus tremuloides*, quaking aspen.

INTRODUCTION

One of the most perplexing questions in the study of plant–insect interactions is why plants vary in their allocation of resources to secondary metabolites. Carbon–nutrient balance (CNB) theory (Bryant et al., 1983; Tuomi et al., 1988) suggests that plant growth and allocation to defense compounds are related to carbon and nutrient availability. In general, this theory asserts that fixed carbon supports a level of growth determined by nutrient supply and that carbon in excess of growth demands can be allocated to storage (e.g., starch) or carbon-based defense compounds (e.g., phenolics). Moreover, allocation to storage and defense compounds is related to nutrient availability in a nonlinear manner. Under conditions of extremely low nutrient availability, photosynthesis and growth are both limited, so little carbon is available for allocation to defense. Increases from low to moderate nutrient availability will increase photosynthesis more strongly than growth, resulting in a surplus of carbon available for secondary metabolites. From moderate to high nutrient availability, carbon assimilation rates become saturated, whereas growth continues to show a strong positive response to nutrient supply. Under these conditions, more photosynthate is required for growth (i.e., strong sink strength), resulting in less carbon available for storage and/or carbon-based allelochemicals. Thus, concentrations of secondary compounds will be very low. In terms of increasing carbon availability (e.g., high light availability), however, CNB theory predicts a positive relationship between carbon assimilation and carbon-based defenses.

Plant photosynthesis, growth, and biomass allocation patterns all respond to resource availability. Both growth and allocation patterns reflect the relative availabilities of light and nutrients. For example, light limitations result in decreased root to shoot ratios, whereas nutrient limitations result in increased root to shoot ratios (Chapin et al., 1987). It is the specific relationship between growth and photosynthesis, however, that is important in determining phytochemical responses.

Our test of CNB theory focused on quaking aspen (*Populus tremuloides*). As an early-successional, rapidly growing species, aspen is especially likely to respond to environmental variation (Bryant et al., 1983; Reichardt et al., 1991; Herms and Mattson, 1992). The defense chemistry of aspen consists mainly of condensed tannins and phenolic glycosides (Lindroth and Hwang, 1996), and levels of these compounds can vary markedly among trees (Lindroth et al., 1987; Hemming and Lindroth, 1995). The degree to which environmental conditions such as light and nutrient availability are responsible for such variation, however, is unknown.

In the Great Lakes region, gypsy moths (*Lymantria dispar*) and forest tent caterpillars (*Malacosoma disstria*) cause substantial defoliation to aspen stands. Although aspen is a preferred host of both insects, their performance is strongly and inversely related to foliar phenolic glycoside concentrations (Hemming and Lindroth, 1995). In addition, concentrations of foliar protein are critically important in the performance of herbivores and are often a limiting factor in the growth of tree-feeding insects (Mattson, 1980). Thus, if environmental conditions influence phenolic glycoside and nitrogen levels in aspen, the susceptibility of aspen to herbivory by gypsy moth and forest tent caterpillars will likely be influenced as well.

We evaluated CNB theory by growing aspen under two levels of light and three levels of nutrient availability. We tested the following hypotheses:

Photosynthesis, Tree Growth and Biomass Allocation

H1. Photosynthesis will be positively related to light availability. Photosynthesis will approach maximum rates at moderate nutrient levels, with only a slight increase from moderate to high nutrient availability.

H2. Tree growth will be positively related to light and nutrient availability.

H3. Allocation to shoots and leaves will be inversely related to light availability, and allocation to roots will be inversely related to nutrient availability.

Foliar Chemistry

H4. Levels of foliar nitrogen will be negatively related to light availability and positively related to nutrient availabilities.

H5. Levels of carbon-based allelochemicals or storage compounds in foliage will be positively related to light availability. Levels of defense or storage compounds will be related to nutrient availability in a nonlinear manner, with moderate nutrient availability resulting in the highest concentrations of these compounds.

Insect Performance

H6. Survival, growth, and feeding efficiencies will improve for insects reared on leaves from trees grown with low light levels and/or high nutrient availability.

METHODS AND MATERIALS

General Experimental Design

The experiment utilized a split-plot design, with light (two levels) as the whole plot and nutrients (three levels) as the subplot. Each light condition was replicated with four independent shade houses, for a total of eight houses, each containing aspen growing under one of three different nutrient regimes. Trees were divided into two sets. One set was used to determine how resource availability altered aspen growth, biomass partitioning, and phytochemistry. All of the foliage from each plant was collected and analyzed to obtain whole canopy responses. The second set was used to determine how tree growth conditions influenced gypsy moth and forest tent caterpillar performance. The insect studies were designed to assess the performance of younger (second instar) and older (fourth instar) larvae, and the growth of forest tent caterpillars in situ. In order to relate insect performance to phytochemical responses, a subset of leaves from trees used for the insect studies was collected for phytochemical analysis.

Tree Growth Conditions

To alter light availability, aspen trees were grown in houses (3.7 m wide × 4.9 m long × 3.0 m high) with shade cloth blocking 85% and 30% of sunlight for low light and high light conditions, respectively. Rather than shade vs. no-shade treatments, cloth with two different transmittances was used to control for the nonlight effects of shade cloth (e.g., air movement, precipitation, and other environmental effects). The shade cloth was placed approximately 0.6 m above the ground on the sides of the shadehouses, providing open space to maximize air circulation. Photosynthetic photon flux density (PPFD) was measured on a mostly clear day (August 6, 1992), over a 1.5-hr period beginning at solar noon. Nine readings were taken for each house and three readings in direct sun with an LI-190B quantum sensor (Li-Cor Inc., Lincoln, Nebraska). PPFDs were 235 ± 15, 1209 ± 49, and 1783 ± 76 (mean ± SD) μmol/m^2/sec for low, high, and full sunlight conditions, respectively. Trees were grown in a 60% sand–40% silt loam topsoil mixture. (Soil analyses indicated 0.058% N, 25.2 ppm P and 65 ppm K, for the 60–40 sand–soil mixture; Soil Plant Analysis Lab, Madison, Wisconsin.) Osmocote (18 : 6 : 12 N-P-K) 8- to 9-month slow-release fertilizer was added to the soil mixture at three recommended application rates (R. Dickson, U.S.

Forest Service, Rhinelander, Wisconsin, personal communication) to achieve low nutrient availability (0 g/liter), moderate nutrient availability (3.5 g/liter), and high nutrient availability (7 g/liter).

In 1991, trees were grown from seeds obtained either from the University of Minnesota North Central Experiment Station (two different sets of full-sib seeds) or collected from aspen trees in the University of Wisconsin Arboretum. Of the total number of trees used, 59% were from one full-sib seed source, 19% from the second full-sib seed source, and the remaining 22% were from the field-collected seeds. All seedlings grew for one growing season in a greenhouse, until dormancy, and then were overwintered (bare-rooted) in cold storage. Trees used for growth and biomass partitioning indices were weighed prior to planting in spring 1992. In order to estimate dry weights for the planted seedlings, we used a regression equation calculated from fresh weight versus dry weight for 22 representative seedlings. Each seedling was planted in a 19-liter pot and randomly assigned to one of the light × nutrient availability treatments. Trees were watered as needed. Additional Osmocote fertilizer was applied to the surface of the soil in spring 1993, at rates previously described, and covered with 2 cm of the sand–soil mixture. Pots were elevated on wooden furring strips to prevent absorption of runoff from neighboring pots.

Photosynthesis, Tree Growth, and Biomass Partitioning

Photosynthetic rates were measured on trees in situ, with an LCA-2 portable infrared gas analyzer and a Parkinson broadleaf chamber (Analytical Development Co., Hoddesdon, U.K.). We took measurements from one to two trees for each nutrient level within each shadehouse. For each tree sampled, we measured a young (fifth position from terminal leaf) and mature (the first fully expanded and dark green) leaf.

Upon completion of the study (mid-June 1993), we harvested six to eight trees from each nutrient level within each shadehouse. Leaves were removed from each tree and chilled (not frozen) on ice until processing later the same day. Leaves were counted and weighed, and leaf area determined with a Li-Cor 3100 leaf area meter (Li-Cor Inc.). Leaves were then flash frozen in liquid nitrogen and freeze-dried. Leaf dry weights were determined and samples were ground in a Wiley mill (40 mesh) and stored in a −20°C freezer prior to chemical analyses. Roots were washed and dried, with stems, at 70°C. Despite washing, about 30% of the dry weight of large root samples was due to adhering sand. We therefore used an alternative weighing procedure for roots. For every root sample, a subsample of dried, homogenized roots was weighed before and after combustion in a muffle furnace. The proportion of weight lost during combustion was multiplied by the original sample weight to obtain an estimate of the organic fraction of the root sample. Mineral fractions of root samples were estimated based on the proportion of ash

TABLE 1. TREE GROWTH AND BIOMASS PARTITIONING PARAMETERS

Parameter	Abbreviation	Calculation
Tree net growth	TNG	ln(total tree dry wt_{final}) − ln(total tree dry $wt_{initial}$)
Root to shoot ratio	RSR	root dry wt/leaf dry wt + stem dry wt
Root weight ratio	RWR	root dry wt/total tree dry wt
Stem weight ratio	SWR	stem dry wt/total tree dry wt
Leaf weight ratio	LWR	leaf dry wt/total tree dry wt
Leaf area ratio	LAR	total leaf area/total tree dry wt
Leaf weight area	LWA	leaf dry wt/total leaf area

remaining (5.37% ± 0.59 SD) from a set of roots from six trees that were carefully cleaned, then combusted. This proportion was added to the organic fraction to obtain the final estimate of root weight. Tree growth and biomass partitioning parameters were calculated as indicated in Table 1.

Foliar Chemistry

To examine light and nutrient availability effects on foliar chemistry, we assayed leaves for nutritional components [water, nitrogen, total nonstructural carbohydrates (TNC)], and allelochemicals (phenolic glycosides and condensed tannins). Water content was measured gravimetrically. We determined Kjeldahl nitrogen by a micro-Nesslerization technique (Lang, 1958) following acid digestion of leaf samples (Parkinson and Allen, 1975). Glycine *p*-toluenesulfonate (5.665% nitrogen) was used as the standard. TNC concentrations (hexoses, sucrose, and starch) were quantified by the procedure of M. M. Schoeneberger, K. Ludovici, and P. Faulkner (unpublished method). Concentrations of the phenolic glycosides tremulacin and salicortin were determined by high-performance thin layer chromatography (Lindroth et al., 1993). Purified phenolic glycosides were used as standards. Condensed tannins were analyzed by the acid butanol technique of Porter et al. (1986), with condensed tannins isolated from quaking aspen as a standard (Hagerman and Butler, 1980).

Insect Studies

To determine how environmentally induced changes in aspen phytochemistry affected herbivores, we reared gypsy moth and forest tent caterpillars on leaves from trees grown under each of the six treatments. Different trees were used for each different type of insect study, which included: a second-instar bioassay, a fourth-instar bioassay, and a forest tent caterpillar in situ growth trial. Each bioassay tree was used to feed both gypsy moth and forest tent caterpillars. Gypsy moth egg masses were provided by USDA-APHIS, Otis Air National Guard Base, Massachusetts, and forest tent caterpillar egg bands were collected

from aspen trees in Menominee County, Wisconsin. Prior to the bioassays, all larvae were reared on aspen foliage collected from trees in the University of Wisconsin Arboretum. In an effort to match first-instar development with early leaf development, the temperature of the rearing incubator was set to mimic field temperatures. After the first stadium, incubators were set at 24°C and 14L:10:D cycle for the insect studies.

Phytochemical Analyses of Bioassay Foliage. Leaf samples were collected from each tree and processed as described previously. Foliage was analyzed for water, nitrogen, phenolic glycosides, and condensed tannins. Because previous research (Hemming and Lindroth, 1995) indicated that TNC did not influence gypsy moth or forest tent caterpillar performance, leaves from bioassay trees were not analyzed for carbohydrates.

Second-Instar Bioassay. Each assay consisted of 10 gypsy moth or 15 forest tent caterpillars placed in a Petri dish (100 × 15 mm) with leaves collected from one of the six light × nutrient treatments. Insects were assigned to treatments at the first sign of molting. Three replicate assays for each nutrient × shadehouse combination were conducted, for a total of 72 assays for each insect species. Fully expanded leaves were collected from the upper crown (lateral or terminal branch) and petioles immediately inserted into Waterpiks to maintain leaf turgor. During the assay, leaves were replaced as needed, at least every third day. Upon completion of the second stadium, percent mortality, weight of newly molted larvae, and duration of the second stadium were recorded. Duration was calculated as the difference between the times when half the larvae had molted into second and third instars, respectively. Leaves for phytochemical analyses were collected on May 16, three days after commencement of the bioassay.

Fourth-Instar Feeding Trial. Each assay consisted of an individual larva placed into a Petri dish (100 × 15 mm) containing an aspen leaf. Five replicate assays were conducted for each nutrient × shadehouse combination, for a total of 120 assays for each insect species. Selection and treatment of leaves were the same as described for the second-instar bioassays. Upon molting to the fifth instar, larvae were frozen, gypsy moth larvae were sexed, and larvae, frass, and remaining leaf tissue were dried (60°C) and weighed. Nutritional indices were calculated from standard formulas (Waldbauer, 1968) except that initial rather than average weights were used to calculate relative growth (RGR) and relative consumption (RCR) rates (Farrar et al., 1989). Leaves for phytochemical analyses were collected May 29, three days after commencement of the trial.

Forest Tent Caterpillar In Situ Growth Study. We reared forest tent caterpillars on shadehouse trees for comparison with the performance of insects on detached leaves under controlled conditions. (Quarantine status in Wisconsin precluded the use of gypsy moths for this assay.) An individual trial consisted of four weighed, newly molted fourth instars enclosed on a branch in a white mesh fabric bag. Three insect trials were conducted for each nutrient × shade-

house combination, for a total of 72 bags. Bags were changed to new branches before larvae depleted food. Larvae were allowed to feed for 16 days, after which they were removed and weighed. Leaf samples for chemical analyses were collected from branches free of larvae, but enclosed in bags, to simulate conditions of leaves used as larval food. Leaves for phytochemical analyses were collected May 30, five days after beginning the experiment.

Statistical Analyses

Data were analyzed by the SAS MIXED procedure (Littell et al., 1996), with the split-plot model as follows:

$$Y_{ijk} = \mu + L_i + E_{ij} + N_k + (LN)_{ik} + e_{ijk}$$

where Y_{ijk} represents the average response of trees in light level i, house j, and nutrient availability k. Light level, nutrient availability, and the light × nutrient interaction $(LN)_{ik}$ were treated as fixed effects, whereas whole plot (E_{ij}) and subplot (e_{ijk}) error terms were considered as random effects. Cell means for each nutrient × shadehouse combination were computed by the MEANS procedure prior to MIXED analyses (SAS Institute, 1989). The means and standard errors reported were computed by LSMEANS.

Because growth and consumption parameters are strongly affected by initial weight, analysis of covariance (ANCOVA) with initial weight as the cofactor is recommended (Raubenheimer and Simpson, 1992). We used the model-fitting procedure for ANCOVA analyses on a split plot experiment as outlined by Littell et al. (1996) for insect average growth (AGR) and consumption (ACR) rates, total consumption, and tree net growth. Initial weight was a significant component of only the model for tree net growth and was therefore included in the analysis of that parameter only. The effect of tree initial weight was the same for all combinations of light and nutrient availability (single slope model). Standard RGR and RCR for insects and final tree dry weight are also presented for comparison with previous studies.

Because gypsy moth growth rates are sex dependent, use of a narrow initial weight range of larvae resulted in 117 females of 120 larvae. Only data from females were used in analyses.

To relate larval performance and foliar chemistry, we used a correlation procedure (CORR) and stepwise multiple regression procedure (STEPWISE, with $\alpha = 0.15$ as the criterion for fitting variables into the model) (SAS Institute, 1989). STEPWISE is a modification of the forward selection procedure, in which variables that have been entered into the model will only remain in the model if they are significant after new variables are added. Correlation and regression analyses used individual tree and insect data rather than treatment means.

RESULTS

Photosynthesis

Photosynthetic rates were strongly influenced by light, but not nutrient, conditions (Figure 1). High light resulted in a 1.8- and 2.4-fold increase in photosynthetic rates for young and mature leaves, respectively. Increased nutrient availability slightly decreased photosynthetic rates for young leaves, but did not influence rates of photosynthesis for mature foliage. Mature leaves exhibited higher photosynthetic rates than young leaves, especially under high light.

Tree Growth

Tree growth was strongly affected by both the direct and interactive effects of light and nutrient availability (Figure 2). The final dry weight of trees increased under high light conditions. Increasing nutrient availability from low to moderate levels resulted in substantial increases in growth, but from moderate to high levels tended to slightly decrease growth. Without nutrient supplementation, high light increased the final weight of trees by only 49%, whereas with nutrient addition, high light increased the final weight of trees by 427%. Patterns of net tree growth and leaf dry weight paralleled results for final tree weight.

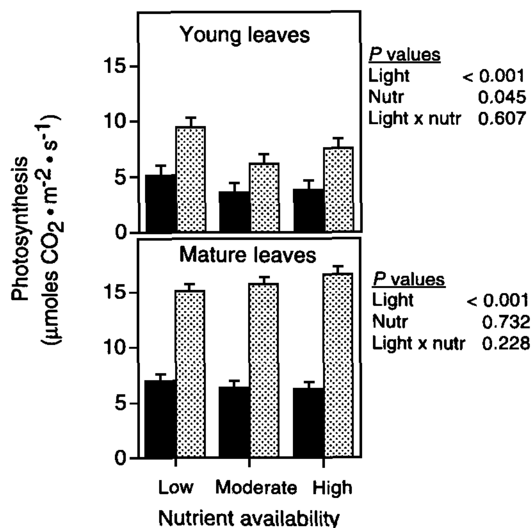

FIG. 1. Responses of aspen photosynthesis to variation in light and nutrient availability ($\bar{X}$ + 1 SE). Dark bars represent low light conditions, light bars represent high light conditions.

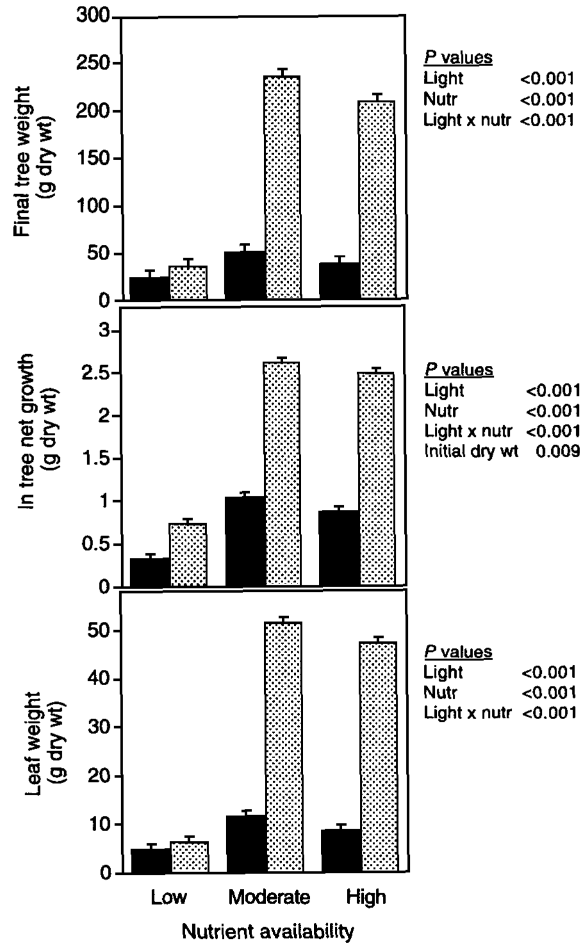

FIG. 2. Aspen growth and leaf mass in response to variation in light and nutrient availability ($\overline{X}$ + 1 SE). Dark bars represent low light conditions, light bars represent high light conditions.

Biomass Partitioning

Light and nutrient availability also altered tree biomass partitioning (Figure 3). Overall, root–shoot ratios increased 68% under high light conditions, but decreased 18% with nutrient supplementation. Root weight ratios paralleled the pattern found for root–shoot ratios. Stem weight ratios increased 25% in low light conditions and showed a slight (marginally significant) increase with

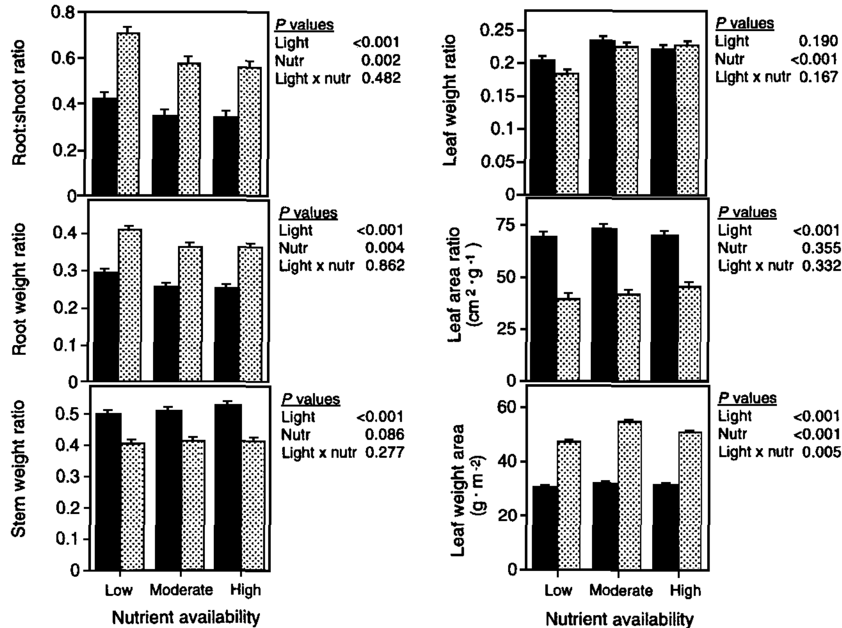

FIG. 3. Aspen biomass partitioning in response to variation in light and nutrient availability ($\overline{X}$ + 1 SE). Dark bars represent low light conditions, light bars represent high light conditions.

increasing nutrient supplementation. Leaf weight ratios were unaffected by light levels, but increased slightly with nutrient supplementation. Although the proportion of biomass allocated to leaves did not differ with respect to light levels, leaf area ratios were 69% greater for trees grown under low light than for those under high light. Leaf weight per unit area (LWA) increased 63% in trees grown under high light conditions. Nutrient supplementation slightly increased LWA, especially under high light conditions.

Foliar Chemistry

Whole Canopy Responses to Light and Nutrient Availability. Foliar nutritional components were influenced by the light and/or nutrient availability of trees (Figure 4). Water content was affected only by light availability, with a 10% decrease in trees grown under high light. Foliar nitrogen declined with increased light, but increased with nutrient supplementation. The difference between nitrogen concentrations in high light versus low light environments was most pronounced for trees grown with low nutrient availability. Hexose

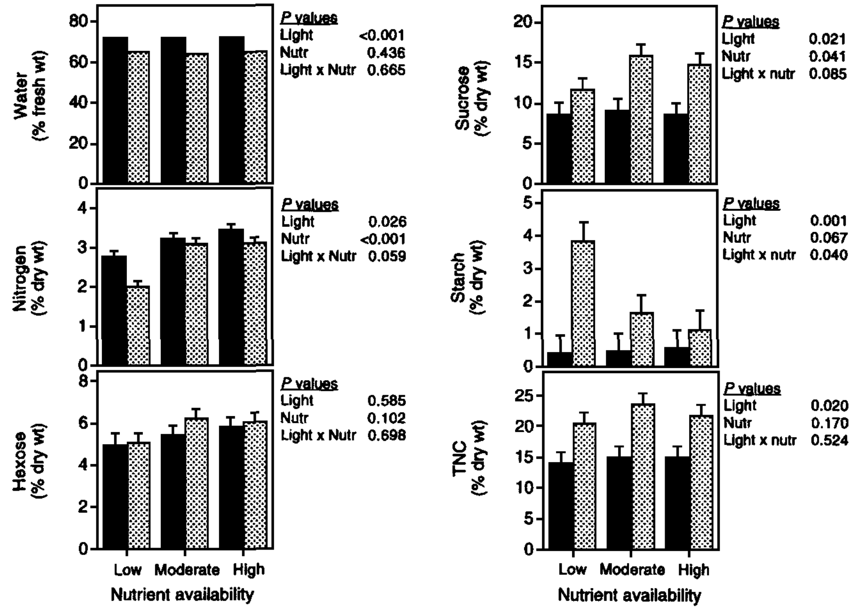

FIG. 4. Concentrations of primary metabolites in aspen foliage in response to variation in light and nutrient availability ($\bar{X}$ + 1 SE). Dark bars represent low light conditions, light bars represent high light conditions.

levels were not significantly altered by resource availability, whereas sucrose levels increased with both light and nutrient availability. Starch concentrations were strongly increased under high light availability. Nutrient supplementation markedly reduced starch levels in high light trees, but had no effect on starch levels in low light trees. Levels of total nonstructural carbohydrates (the combination of hexoses, sucrose and starch) were affected only by light availability, with a 51% increase in foliage grown under high light.

Levels of phenolic compounds were influenced by light and nutrient availability (Figure 5). Concentrations of condensed tannins increased under high light. Under low light, nutrient addition did not alter tannin levels, whereas under high light, nutrient addition markedly reduced tannin levels. With respect to phenolic glycosides, concentrations of tremulacin were more strongly affected by light (a 34% increase in high light), than were those of salicortin. Nutrient availability was a significant factor only for salicortin, levels of which decreased slightly with fertilization. Combined storage plus defense compounds (starch + condensed tannins + phenolic glycosides) (Figure 6) were strongly affected by the direct and interactive effects of light and nutrient availability. Under low

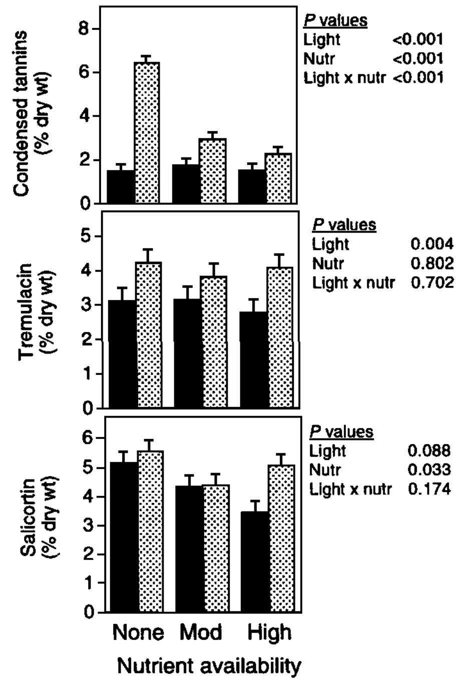

FIG. 5. Concentrations of secondary metabolites in aspen foliage in response to variation in light and nutrient availability ($\bar{X}$ + 1 SE). Dark bars represent low light conditions, light bars represent high light conditions.

light, nutrient effects were minimal. Under high light, increasing nutrient availability from low to moderate sharply decreased amounts of storage and defense compounds, whereas increasing nutrient availability from moderate to high did not alter allocation to those compounds.

Concentrations of phytochemicals showed substantial covariation (Table 2). Water and nitrogen contents were positively correlated. Levels of both water and nitrogen were significantly and inversely related to phenolic glycoside, condensed tannin, and TNC concentrations. Levels of tannins and TNC were positively related. Phenolic glycoside concentrations, however, were not significantly related to levels of condensed tannins or TNC.

Foliar Chemistry from Insect Trials. Phytochemical responses to light and nutrient availability were generally consistent among whole canopy and insect trials. Phenolic glycoside content was consistently elevated in response to high light in all collections, but only the leaves collected for the second-instar trial responded to nutrient availability, with levels declining with nutrient supplemen-

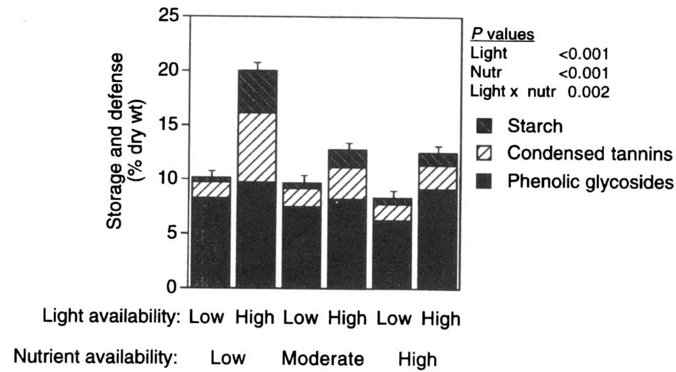

FIG. 6. Concentrations of storage and defense compounds (sum of starch, condensed tannins and phenolic glycosides) in aspen foliage in response to variation in light and nutrient availability ($\overline{X}$ + SE).

tation. Because the different foliage collections spanned a period of about five weeks, some seasonal declines in foliar water and nitrogen levels were observed. The patterns of covariation of foliar compounds were the same for the insect studies as they were for the whole canopy studies, with one exception. Phenolic glycoside and condensed tannin concentrations were positively related in foliage collections made for the second and fourth instar studies.

Insect Trials

Second-Instar Bioassay. Light and nutrient availability of trees only marginally altered the performance of young gypsy moth and forest tent cater-

TABLE 2. CORRELATIONS AMONG FOLIAR CHEMICAL COMPONENTS FROM WHOLE CANOPY HARVEST[a]

	Water	Nitrogen	Total nonstructural carbohydrates	Tannins
Nitrogen	0.35 (<0.001)	1.00		
Total nonstructural carbohydrates	−0.67 (<0.001)	−0.23 (0.007)	1.00	
Tannins	−0.51 (<0.001)	−0.56 (<0.001)	0.29 (<0.001)	1.00
Phenolic glycosides	−0.31 (<0.001)	−0.41 (<0.001)	−0.07 (0.392)	0.10 (0.248)

[a]Values represent r (P).

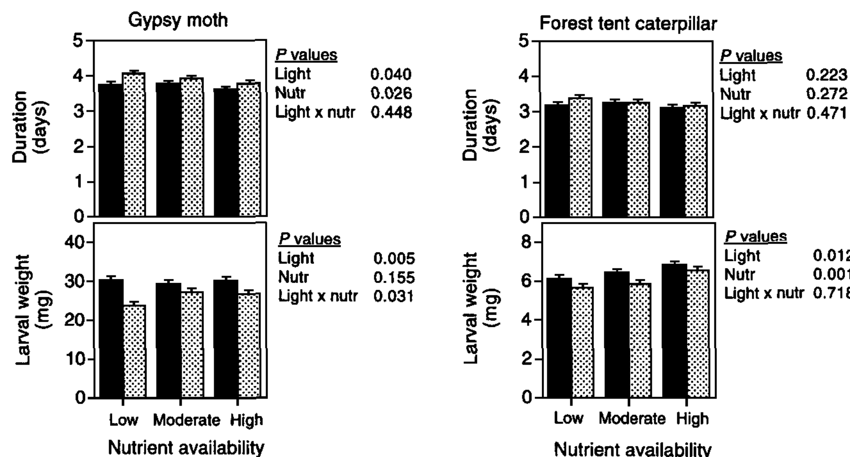

FIG. 7. Performance of second-instar gypsy moths and forest tent caterpillars reared on aspen foliage grown under conditions of differing light and nutrient availability ($\overline{X}$ + 1 SE). Larval weight indicates average fresh weight of newly molted third instars. Dark bars represent low light conditions, light bars represent high light conditions.

pillars (Figure 7). Both species had very low mortality, which was not affected by light or nutrient enrichment (data not shown). Development times increased slightly (5%) and decreased slightly (5%) for gypsy moth larvae reared on high light and nutrient supplemented foliage, respectively. Treatments did not alter development times for forest tent caterpillars. Weights of newly molted third instars were reduced for larvae of both species reared on foliage grown under high light. Forest tent caterpillar weights increased when larvae were reared on leaves from high nutrient treatments. Light and nutrient availability interacted to influence gypsy moth weight; nutrient supplementation increased larval weight only under low light conditions.

Multiple regression analyses revealed that phenolic compounds were the only components associated with variation in gypsy moth weight, with phenolic glycosides accounting for a majority (50%) of the variation (Table 3). Variation in development time for gypsy moth and forest tent caterpillars and weights of forest tent caterpillars was explained exclusively by phenolic glycosides, although they explained less than 10% of the variation in each case (Tables 3 and 4).

Fourth-Instar Bioassay. Resource-mediated changes in aspen foliage had no influence on development times or growth of fourth instar gypsy moth larvae but did alter forest tent caterpillar performance (Figure 8). For forest tent caterpillars, the duration of the fourth stadium increased for larvae reared on foliage

TABLE 3. STEPWISE MULTIPLE REGRESSION ANALYSES OF RELATIONSHIPS BETWEEN SECOND-INSTAR GYPSY MOTH PERFORMANCE AND FOLIAR CHEMISTRY[a]

	Regression model			Partial regression components		
Parameter	Equation	r^2	P	Variable	r^2	P
Duration	$Y = 0.04(PG) + 3.6$	0.064	0.032	[b]		
Weight	$Y = -0.6(PG)$	0.538	<0.001	PG	0.495	<0.001
	$- 1.1(CT) + 35.1$			CT	0.043	0.013

[a] $N = 72$, $\alpha = 0.15$ was used as the criterion for fitting components into the model. PG = total phenolic glycosides, CT = condensed tannins.
[b] Regression coefficients for models with single independent variables are given by the full model.

from high light trees. Increased nutrient availability reduced stadium duration, especially for insects fed high light foliage. Growth rates for both species were lowest when larvae were reared on the high light–low nutrient treatment foliage, although this was only significant for forest tent caterpillars.

Resource-mediated changes in aspen foliage had relatively little influence on gypsy moth consumption (Figure 9). Total consumption by gypsy moths increased for larvae reared on high light foliage and declined slightly for larvae reared on nutrient supplemented foliage. Forest tent caterpillars exhibited a stronger consumption response to resource-mediated changes in aspen foliage than did gypsy moths. Under low light conditions, consumption responses were similar across nutrient availabilities. In contrast, under high light conditions, consumption was reduced on low nutrient foliage and elevated on moderate and high nutrient foliage.

Approximate digestibility (AD) was affected similarly for both gypsy moth and forest tent caterpillars, with values highest for insects on high light–nutrient treatments and similar for all other treatments (Figure 10). Food conversion efficiencies of forest tent caterpillars were affected more than those of gypsy moths,

TABLE 4. STEPWISE MULTIPLE REGRESSION ANALYSES OF RELATIONSHIPS BETWEEN SECOND-INSTAR FOREST TENT CATERPILLAR PERFORMANCE AND FOLIAR CHEMISTRY[a]

	Regression model		
Parameter	Equation	r^2	P
Duration	$Y = 0.04(PG) + 3.0$	0.099	0.008
Weight	$Y = -0.10(PG) + 6.8$	0.084	0.014

[a] $N = 72$. $\alpha = 0.15$ was used as the criterion for fitting components into the model. PG = total phenolic glycosides. Only phenolic glycosides met the acceptance criterion.

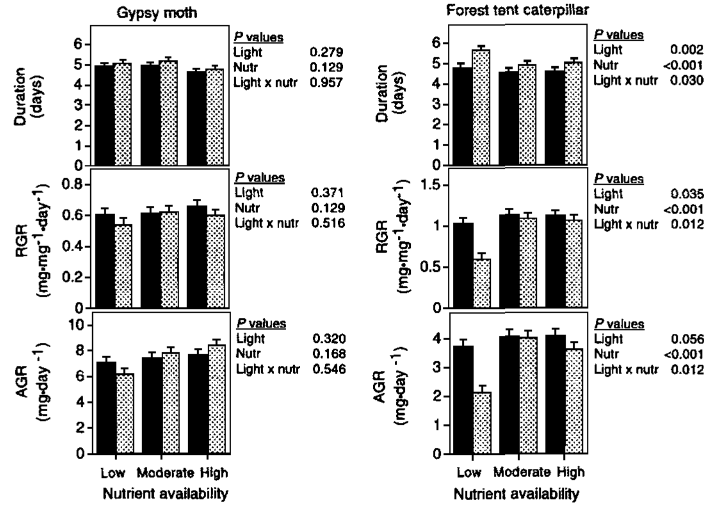

FIG. 8. Growth of fourth instar gypsy moths and forest tent caterpillars reared on aspen foliage grown under conditions of differing light and nutrient availability ($\bar{X}$ + 1 SE). RGR = relative growth rate, AGR = average growth rate. Dark bars represent low light conditions, light bars represent high light conditions.

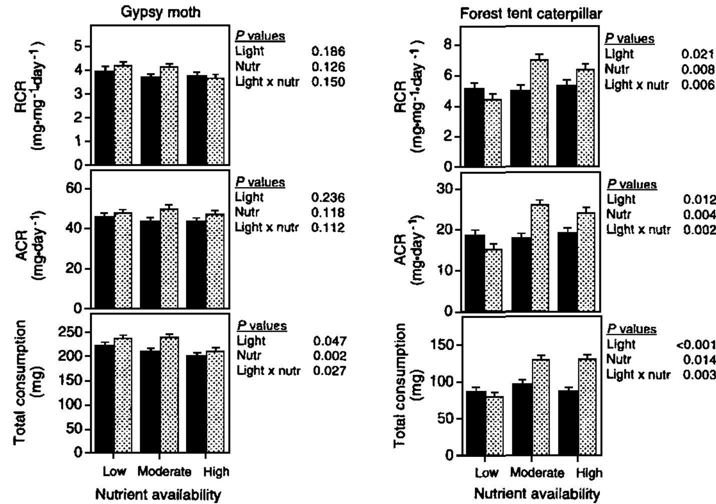

FIG. 9. Consumption by fourth instar gypsy moths and forest tent caterpillars reared on aspen foliage grown under conditions of differing light and nutrient availability ($\bar{X}$ + 1 SE). RCR = relative consumption rate, ACR = average consumption rate. Dark bars represent low light conditions, light bars represent high light conditions.

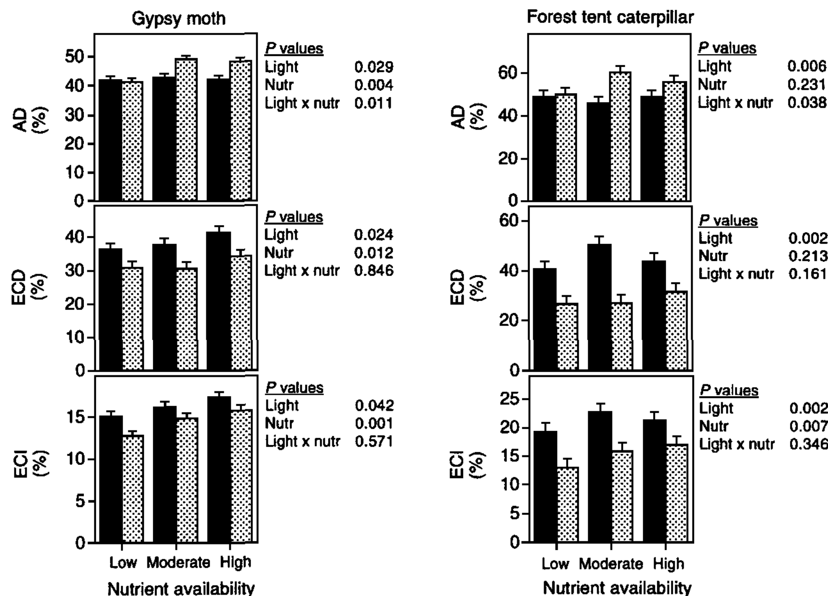

FIG. 10. Approximate digestibility (AD), efficiency of conversion of digested food (ECD), and efficiency of conversion of ingested food (ECI) for fourth-instar gypsy moths and forest tent caterpillars reared on aspen foliage grown under conditions of differing light and nutrient availability ($\overline{X}$ + 1 SE). Dark bars represent low light conditions, light bars represent high light conditions.

although patterns were generally similar. For both species, efficiencies of conversion of digested food (ECDs) decreased in larvae reared on leaves from high light treatments. Additionally, gypsy moth ECDs increased slightly in larvae fed leaves from nutrient supplemented treatments. High light foliage decreased, whereas nutrient supplemented foliage increased, the efficiency with which both species converted ingested food into biomass (ECI).

Stepwise multiple regression analyses of gypsy moth performance revealed that phenolic glycoside levels accounted for the highest proportion of the variation in stadium duration, RGR, AGR, ACR, and ECI (Table 5). Variation in gypsy moth RCR was associated with both condensed tannin and phenolic glycoside concentrations, and total consumption was associated most strongly with condensed tannin concentrations. Variations in AD and ECD were most strongly accounted for by water content, although condensed tannin levels also accounted for some of the variation in AD.

For forest tent caterpillars, water content explained the highest proportion of the variation in stadium duration, total consumption, AD, ECD, and ECI (Table 6).

TABLE 5. STEPWISE MULTIPLE REGRESSION ANALYSES OF RELATIONSHIPS BETWEEN FOURTH-INSTAR GYPSY MOTH PERFORMANCE AND FOLIAR CHEMISTRY[a]

	Regression model			Partial regression components		
Parameter	Equation	r^2	P	Variable	r^2	P
Duration	$Y = 0.1(PG) + 0.07(CT) + 4.0$	0.306	<0.001	PG	0.289	<0.001
				CT	0.017	0.100
RGR	$Y = -0.02(PG) + 0.05(N) + 0.65$	0.294	<0.001	PG	0.270	<0.001
				N	0.024	0.051
AGR	$Y = -0.3(PG) + 0.6(N) + 7.9$	0.318	<0.001	PG	0.298	<0.001
				N	0.020	0.068
RCR	$Y = 0.2(CT) - 0.09(PG) + 4.3$	0.267	<0.001	CT	0.145	<0.001
				PG	0.121	<0.001
ACR	$Y = -1.2(PG) + 2.2(CT) + 51.7$	0.282	<0.001	PG	0.152	<0.001
				CT	0.130	<0.001
TC	$Y = 14.8(CT) - 2.1(PG) + 215.5$	0.250	<0.001	CT	0.222	<0.001
				PG	0.028	0.041
AD	$Y = -0.9(H_2O) - 1.5(CT) + 2.4(N) + 103.1$	0.311	<0.001	H_2O	0.139	<0.001
				CT	0.134	<0.001
				N	0.038	0.014
ECD	$Y = 0.6(H_2O) - 0.6(PG) + 2.7(N) - 11.0$	0.304	<0.001	H_2O	0.225	<0.001
				PG	0.055	0.004
				N	0.024	0.052
ECI	$Y = -0.3(PG) + 2.0(N) + 0.1(H_2O) + 5.4$	0.400	<0.001	PG	0.246	<0.001
				N	0.145	<0.001
				H_2O	0.013	0.117

[a]($N = 117$). $\alpha = 0.15$ was used as the criterion for fitting components into the model. PG = phenolic glycosides, N = nitrogen, CT = condensed tannins, H_2O = water. RGR = relative growth rate, AGR = average growth rate, RCR = relative consumption rate, ACR = average consumption rate, TC = total consumption, AD = approximate digestibility, ECD = efficiency of conversion of digested food, ECI = efficiency of conversion of ingested food.

TABLE 6. STEPWISE MULTIPLE REGRESSION ANALYSES OF RELATIONSHIPS BETWEEN FOURTH-INSTAR FOREST TENT CATERPILLAR PERFORMANCE AND FOLIAR CHEMISTRY[a]

Parameter	Regression model			Partial regression components		
	Equation	r^2	P	Variable	r^2	P
Duration	$Y = -0.03(H_2O) + 0.3(N) + 0.06(PG) + 0.08(CT) + 7.3$	0.418	<0.001	H_2O	0.240	<0.001
				N	0.093	<0.001
				PG	0.069	<0.001
				CT	0.016	0.091
RGR	$Y = 0.2(N) - 0.07(CT) + 0.4$	0.345	<0.001	N	0.282	<0.001
				CT	0.064	0.002
AGR	$Y = 0.8(N) - 0.2(CT) + 1.8$	0.316	<0.001	N	0.249	<0.001
				CT	0.067	0.002
RCR	$Y = -0.4(CT) - 0.2(H_2O) + 0.5(N) + 17.2$	0.222	<0.001	CT	0.137	<0.001
				H_2O	0.059	0.011
				N	0.027	0.060
ACR	$Y = -1.6(CT) - 0.6(H_2O) + 1.6(N) + 62.4$	0.213	<0.001	CT	0.142	<0.001
				H_2O	0.052	0.017
				N	0.019	0.114
TC	$Y = -3.7(H_2O) - 8.1(CT) + 1.1(PG) + 367.7$	0.337	<0.001	H_2O	0.198	<0.001
				CT	0.124	<0.001
				PG	0.015	0.122
AD	$Y = -1.3(H_2O) - 1.3(CT) -0.5(PG) + 147.3$	0.341	<0.001	H_2O	0.270	<0.001
				CT	0.039	0.015
				PG	0.032	0.026
ECD	$Y = 1.9(H_2O) + 5.4(N) - 113.1$	0.607	<0.001	H_2O	0.562	<0.001
				N	0.045	<0.001
ECI	$Y = 0.7(H_2O) + 3.3(N) - 38.1$	0.648	<0.001	H_2O	0.531	<0.001
				N	0.117	<0.001

[a] $N = 110$, $\alpha = 0.15$ was used as the criterion for fitting components into the model. N = nitrogen, CT = condensed tannins, H_2O = water, PG = phenolic glycosides. RGR = relative growth rate, AGR = average growth rate, RCR = relative consumption rate, ACR = average consumption rate, TC = total consumption, AD = approximate digestibility, ECD = efficiency of conversion of digested food, ECI = efficiency of conversion of ingested food.

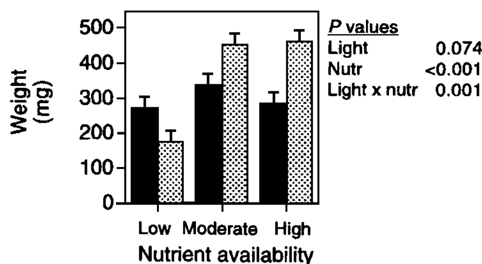

FIG. 11. Fresh weight of forest tent caterpillars (FTC) reared in situ on aspen under various light and nutrient availability treatments ($\overline{X}$ + 1 SE). Dark bars represent low light conditions, light bars represent high light conditions.

The most important components accounting for variation in growth rates and consumption rates were nitrogen and condensed tannins, respectively. Phenolic glycoside concentrations were not strongly associated with variation in forest tent caterpillar performance parameters.

Forest Tent Caterpillar In Situ Growth Study. The final weights of forest tent caterpillar larvae, reared for 16 days in the shadehouses, were strongly influenced by resource availability of the trees (Figure 11). Under low light conditions, nutrient treatments had little effect on larval weights. Under high light conditions, however, increasing nutrient availability from low to moderate levels resulted in a 2.6-fold increase in larval weights. Further increase in nutrient availability did not affect larval weights. Stepwise multiple regression analysis revealed that 39% of the variation in the weight of larvae could be explained with a model that included all of the chemical parameters measured, although nitrogen explained the highest proportion (22%) of variation.

DISCUSSION

Photosynthesis, Tree Growth, and Biomass Allocation

Increasing light availability increased photosynthetic rates, as expected (H1). Contrary to H1, photosynthetic rates were unaffected by nutrient availability. Photosynthesis is generally correlated with leaf nitrogen content (Chapin et al., 1987). As photosynthetic rates were similar across nutrient regimes, despite a 35% reduction in nitrogen concentrations in high light, unfertilized foliage, leaf nitrogen levels were apparently above a threshold affecting photosynthesis. Bowman and Conant (1994) found that in the second year of a nitrogen supplementation study, *Salix glauca* responded to fertilization by producing more leaves per shoot than did unfertilized trees, without altering photosynthetic rates

per unit leaf area. The general response of aspen appears to be similar in maintaining photosynthetic rates while altering leaf production. If this is indeed the case, allocation to storage and defense compounds would typically decline in response to nutrient addition, rather than increase from low to moderate nutrient availability as predicted by CNB theory (Tuomi et al., 1988). Only when nutrient availability is so low as to result in reduced photosynthetic rates would nutrient supplementation lead to increases in allocation to storage and defense compounds.

Increasing resource availability—both light and nutrients—promoted tree growth, consistent with H2. Because aspen is a shade-intolerant species, the dramatic increase in growth in high light conditions is not surprising. Low nutrient availability limited growth under low and high light conditions, but nutrient supplementation resulted in a proportionally greater increases in growth for trees in high light environments. Growth exhibited a nonlinear response to nutrient supply, most likely because nutrient uptake was effectively saturated at the moderate fertilization levels.

As predicted by H3, trees tended to allocate biomass to tissues that maximize the acquisition of limiting resources (Chapin, 1980; Chapin et al., 1987). Under low light, trees increased allocation to shoots, a common response in studies with early successional tree species (e.g., Bazzaz and Miao, 1993; Walters et al., 1993; Walters and Reich, 1996). Leaf weight ratios were not significantly changed by light. Leaf area ratios, however, increased greatly in trees grown under low light, suggesting that aspen enhances its light-harvesting capacity by increased leaf area, without a significant change in biomass allocated to leaves. Finally, trees grown with low nutrient availability increased allocation of biomass to roots, results consistent with numerous other studies (e.g., McDonald et al., 1986; Bazzaz and Miao, 1993; Pettersson et al., 1993; El Kohen and Mousseau, 1994; but see Brown and Higgenbotham, 1986; Kinney and Lindroth, 1997).

Foliar Chemistry

As predicted by H4, high light conditions reduced foliar nitrogen concentrations. Such decreases are commonly observed under conditions of high carbon availability such as high light (e.g., Bryant, 1987; Hartley et al., 1995; Ruohomäki et al., 1996; Lawler et al., 1997) or enriched CO_2 (reviewed in McGuire et al., 1995). This is partly due to "nitrogen dilution" by accumulating carbon-based compounds (e.g., starch, phenolics). Increased concentrations of starch and phenolics and increased LWA in high light–low nutrient availability treatments provide support for this interpretation. As expected, increasing nutrients from low to moderate levels significantly increased foliar nitrogen, although no difference was found between moderate and high availability treatments. The high-

light-mediated decrease in foliar nitrogen levels was greatest under low nutrient availability. This indicates that nutrient addition decreased the carbohydrate source–sink ratio, enhancing growth, and increasing foliar nitrogen concentrations.

In general, concentrations of storage and defense compounds followed the pattern predicted by CNB theory. Growth in high light conditions led to increased levels of starch and phenolics (H5), findings consistent with numerous other studies (reviewed by Koricheva et al., 1998). Although responses to nutrient availability were not consistent with our prediction (H5), photosynthesis and growth data indicate that the patterns of variation in carbon-based storage and defense compounds were consistent with CNB theory. In high light environments, the response to nutrient availability is strongly dependent on relative sink strength. Low nutrient trees maintained similar photosynthetic rates to fertilized trees, yet were severely growth limited. CNB theory predicts an increase in concentrations of storage and defense compounds under such conditions, because low sink strength favors accumulation of carbohydrates. Indeed, levels of such compounds were highest in the high light–low nutrient treatment. Increasing nutrient availability promoted growth, increasing sink strength and reducing carbon accumulation. Because increasing nutrient availability from moderate to high levels did not change leaf nitrogen or photosynthetic or growth rates, the similar levels of starch and phenolics in nutrient supplemented treatments are consistent with predictions of CNB theory. The interactive effect of light and nutrient availability on storage and defense levels is also expected. In low light, nutrient availability has minimal effect on concentrations of storage and defense compounds because carbon limits growth. However, when carbon availability is high, strong responses to nutrient availability are predicted.

Overall, levels of condensed tannins respond to changes in resource availability more strongly than do levels of phenolic glycosides (e.g., Bryant et al., 1987; Roth and Lindroth, 1995; Kinney et al., 1997; Roth et al., 1997; McDonald and Lindroth, 1999). The differences in allocation patterns may be due to differences in rates of synthesis or degradation. Phenylalanine ammonium lyase (PAL) deaminates the amino acid phenylalanine, forming precursors for phenolic synthesis. PAL is regulated by a number of different developmental, physiological, and environmental factors (reviewed in Matsuki, 1996), and therefore may play a role in the response of phenolics to resource availability. Because condensed tannins and phenolic glycosides respond differently to resource availability, their synthesis may be independently regulated, at steps subsequent to PAL activity. Alternatively, if these compounds differ in degradation rates, a common synthetic regulation mechanism would not be precluded. Reichardt et al. (1991) proposed that "static" compounds, those with low turnover rates (e.g., tannins), are more likely to follow the pattern of variation predicted by CNB theory than are "dynamic" compounds, those that turn over (e.g., phenolic glycosides).

Insect Responses

In general, differences in phytochemistry did not translate into pronounced treatment effects on insect performance. In comparison with other studies of gypsy moth and forest tent caterpillars reared on aspen (Hemming and Lindroth, 1995; Hwang and Lindroth, 1997), the high growth rates and short stadium durations observed in this study suggest that overall insect performance was good. Although treatment differences were slight, in both species and for all developmental stages tested, growth was lowest and stadium duration was longest when larvae ate leaves from the high light–low nutrient trees, consistent with H6.

Resource availability modified growth of gypsy moths only as second instars. While small changes in food conversion efficiencies were observed for fourth instars, they had little or no effect on growth and consumption and are therefore unlikely to have ecological relevance. Previous research indicates that gypsy moths are strongly influenced by phenolic glycosides (Hemming and Lindroth, 1995; Wang and Lindroth, 1997). The unexpected similarity of performance among insects on different treatments in this study likely occurred because phenolic glycoside levels were low to moderate and did not respond substantially to variations in resource availability. That phenolic glycoside levels likely had some effect on gypsy moths is suggested by results of the regression analyses.

Considering that forest tent caterpillars are usually less susceptible to variation in aspen phytochemistry than are gypsy moth caterpillars (Hemming and Lindroth, 1995; Hwang and Lindroth, 1997), it is surprising that the most dramatic difference in insect performance was the growth reduction of fourth-instar forest tent caterpillars on high light–low nutrient foliage. According to multiple regression analyses, the reduction of performance on that treatment may have been due to the decrease in foliar nitrogen. Hemming and Lindroth (1995) also found a strong relationship between forest tent caterpillar performance and foliar nitrogen. Thus, forest tent caterpillars appear to be less tolerant of low nitrogen levels, and more tolerant of phenolic glycosides, than are gypsy moths.

Forest tent caterpillars consistently responded to treatment induced changes in foliar quality by altering their feeding behavior. Although fourth instars increased consumption on leaves from the high light–nutrient-supplemented treatments, herbivory is less consequential for trees growing in such environments. Compared with the high light–low nutrient treatment, the 59% increase in consumption on the high light–fertilized trees in more than compensated for by the 700% increase in leaf mass.

The pattern of forest tent caterpillar growth in situ was similar to that of fourth instars reared on detached leaves in controlled environmental chambers. Insects did most poorly when reared on high light–low nutrient foliage. However, growth of insects in situ was enhanced when they were reared on high

light–nutrient-supplemented trees, whereas growth of fourth instars (in incubators) did not vary between high and low light in the nutrient-supplemented treatments. The growth enhancement observed in situ was most likely due to warmer temperatures (by 2–4°C) in high light houses.

Overall, responses of aspen to a large range of light and nutrient availabilities resulted in minimal effects on gypsy moth and forest tent caterpillar performance. The high light–low nutrient treatment, however, consistently reduced insect performance. Given that aspen grows over a wide range of environmental conditions, its interactions with insects are likely influenced by resource availability in some habitats. In addition, research conducted following this study revealed that variation in aspen phytochemistry has a strong genetic component, which in turn strongly influences insect performance (Hwang and Lindroth, 1997). Research now in progress seeks to clarify whether resource availability and genotype interact to influence phytochemical and insect responses.

Implications for CNB Theory

Patterns in accumulations of phytochemicals in response to changes in resource availability were generally consistent with predictions of CNB theory. However, low light trees allocated over 8% dry leaf mass to storage and defense compounds even though growth of these trees was carbon limited. Additionally, high light–high nutrient trees allocated 12% of dry leaf mass to starch and phenolics even though growth of these trees indicated that nutrient uptake was saturated. Such a large allocation to carbon-based compounds under conditions of low source strength or high sink strength [i.e., "basal" allocation (sensu McDonald and Lindroth, 1999)] is incompatible with CNB theory, which posits that resources are only allocated to storage and defense when growth demands for carbon have been met (Tuomi et al., 1988).

Interestingly, the pattern of accumulation of excess carbon (allocation after growth demands for carbon have been met) differed dramatically from the pattern of allocation of basal secondary metabolites (allocation before growth demands for carbon have been met). Excess carbon accumulated as starch and condensed tannins. According to CNB theory, the allocation of excess carbon reflects evolutionary responses of plants to herbivores, yet aspen appears to allocate surplus carbon to ineffective defenses, at least against the insects we tested. The allocation of excess carbon to starch allows flexibility, as it can be used for energy production, converted to other substances, or mobilized for transport. The allocation of excess carbon to condensed tannins may be a genetic trait resulting from selection pressure exerted by other herbivores or pathogens, or may reflect the utility of condensed tannins for nondefense purposes.

In contrast, the largest proportion of basal secondary metabolites consisted of phenolic glycosides, an effective defense against multiple aspen pests (Lin-

droth and Hwang 1996). CNB theory provides no framework with which to interpret these findings, as basal allocation is unexpected. A subsequent theory, growth–differentiation balance (GDB) (Herms and Mattson, 1992), explains physiological responses within a larger context of life history and evolutionary considerations. GDB theory allows for evolutionary trade-offs between growth and defense to occur (growth does not always have priority for resources). For example, under conditions where competition is the primary selective force, plants preferentially allocating resources to growth over defense would be selected. However, in environments where herbivory is a strong selective force, plants allocating resources to defenses at the expense of growth would be selected. Thus, basal allocation appears to represent a case where a trade-off between growth and defense has occurred (Mole, 1994), as the diversion of carbon (under conditions of low source or high sink strength) to phenolic compounds may exact a cost in terms of reduced growth rate. Although CNB theory provides a useful framework to predict allocation to carbon-based storage and defense compounds, the inclusion of the role of selective pressures in the evolution of plant defenses makes GDB theory a more complete model with which to understand the evolution of plant defense chemistry.

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Book Review

The Insects: Structure and Function, 4th ed. R. F. Chapman. New York: Cambridge University Press, 1998. U.S. $130.00 (hardback, ISBN 0-521-57048-4), $54.95 (paperback, ISBN 0-521-57890-6).

This newest edition of a classic book on the anatomy and physiology of insects will be welcomed by the wide audience already aware of the excellent merits of its preceding editions. The beauty of this volume for chemical ecologists is that it forms a ready reference to the structures and overall physiology of each of the systems of the insect organism, and the level of the text does not presume any previous entomological training. As such, this well-organized reference work should occupy a place in the library of every chemical ecologist working with insects.

What does this volume have that makes it particularly useful to a chemical ecologist? In the first place, the book is logically organized by major insect parts dealing with particular functions (e.g., the thorax and locomotion). Within each functional section are a series of chapters, each covering both the structures and physiology relevant to that particular overall function (e.g., legs, wings, sensilla on the halteres, muscular control). Thus, if one were interested in communication, one would go to Part V, "Communication," and then decide whether one wanted to look at physiological coordination within the insect, perception of the environment, or communication with other organisms. If interested in colors and insects, one would choose the last section and go immediately to the first of three chapters in that section, which deals with Visual Signals: Color and Light Production, and has a specific section in the Table of Contents identified as 25.4, The Colors and Insects. Here one would find a series of subtitled textual statements summarizing important information on color patterns in butterflies, physiological color change, morphological color change, and ontogenetic changes, as well as other factors affecting color. Ample reference is made to the original literature so that one can pursue virtually any topic in more detail by going to the References Cited at the end of each chapter.

The book is superbly illustrated with line drawings, graphs, and occasional halftone photographs. The drawings are clearly rendered and adequately labeled so that even the novice examining the reproductive system of an insect for the

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first time can find the various parts in the diagrams which may be of particular interest to a problem that he or she is researching.

The text itself is written very clearly and one can recommend this to either undergraduate or graduate students without any hesitation whatsoever. The abundant literature citations and excellent index add to the value of the textual discussions, making this book an invaluable resource for any biologist working with insects and aspects of their chemical ecology and physiology.

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CHEMICAL STIMULI FROM APPLE INFLUENCE THE BEHAVIOR OF NEONATE CATERPILLARS OF THE GENERALIST HERBIVORE, *Epiphyas postvittana*

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Abstract—When introduced into a wind tunnel with low windspeeds (0.2–0.3 m/sec), neonate *E. postvittana* larvae were more likely to walk in a downwind rather than upwind direction. This tendency to walk downwind did not change when odors from apple leaves or fruit were introduced into the wind tunnel. In a second assay that measured travel times of larvae as they walked from the center to the edge of filter paper disks, larvae moved more slowly on disks treated with extracts of apple leaves or extracts of apple fruit, but did not slow their movement on disks treated with extracts of a nonhost, *Coprosma repens*. Analysis of videorecords revealed that larvae on disks treated with a dichloromethane extract of apple leaves took more circuitous routes, walked more slowly, and stopped more frequently than larvae walking on solvent-treated disks. When the dosage of this dichloromethane apple leaf extract was increased or when larvae were held without food prior to testing, differences between travel times on solvent- and extract-treated disks did not increase significantly. The dichloromethane apple leaf extract, when tested in the wind tunnel with low windspeeds, also caused larvae to delay spinning down on a silken thread after reaching the edge of the disk, but had no effect on spin-down times when tested in still-air conditions. Testing of rotary evaporated apple leaf extracts and fractionation of these extracts indicated that a number of both volatile and relatively involatile chemicals contribute to the behavioral responses of *E. postvittana* larvae.

Key Words—Walking, dispersal, epicuticular waxes, volatiles, host-finding.

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INTRODUCTION

In recent decades, a great deal of research effort has been devoted to understanding how herbivorous insects find and identify their plant hosts (Bernays and Chapman, 1994; Schoonhoven et al., 1998). Common subjects of this research have been holometabolous insect species that feed on plant tissue as larvae but not as adults. For some species in this group, adult females move from plant to plant as they oviposit, but the neonate larva cannot move from the plant it has been placed on by its mother (e.g., many herbivorous flies, some lepidopteran species). In contrast to these insect species are those species that have both mobile adult females and mobile neonate larvae (e.g., other lepidopteran spp.). Here both the adult female and larva may contribute to host finding and identification.

We are investigating the relative contributions of adult females and neonate larvae to host-finding and identification in the generalist species, the light-brown apple moth, *Epiphyas postvittana* (Walker) (Lepidoptera: Tortricidae). While the notoriety of this insect is due to its pest status on Australian and New Zealand apples, its host range is large, encompassing in excess of 250 species in more than 50 plant families (Danthanarayana, 1975; Wearing et al., 1991). After mating, the adult female typically lives for several weeks and over that period oviposits up to 1200 eggs in a series of clutches ranging in size from 3 to 150 eggs (Geier and Briese, 1980; Wearing et al., 1991). Larvae hatching from these egg clutches signal their acceptance of a plant by spinning a silken shelter from which feeding occurs. However, before such shelters are constructed, larvae generally move away from the egg mass by walking and/or by spinning down on a silken thread (Geier and Briese, 1980).

In *E. postvittana*, an understanding of the relative contributions of adult females and neonate larvae to host-finding and identification is starting to emerge. In terms of the correspondence between adult female oviposition preferences and larval feeding preferences, it appears that neonate larvae do not necessarily abide by the choice of the ovipositing female. When given a choice of 26 plant species (Foster and Howard, 1999), *E. postvittana* adult females exhibited little or no ovipositional preference among species that have been recorded as hosts or nonhosts in pest surveys of New Zealand natural and cultivated systems. In contrast, neonate larvae were more likely to settle and feed on recorded host plants than on nonhosts. This suggests that *E. postvittana* host preferences may be largely governed by the behavior and sensory abilities of the neonate larva.

Previous studies have shown that information gathered by a variety of sensory systems influences the movement and settling behavior of neonate *E. postvittana* larvae. Visual information influences the direction that larvae move when walking and spinning-down (Harris et al., 1995). Walking larvae move

towards yellow-green targets that subtend as little as 5–8 degrees but, in the absence of such targets, move towards targets with low reflectance (i.e., dark areas). Spinning down on a silken thread is also influenced by color, being inhibited when the substrate beneath the larva is blue as opposed to brown or green. Once a larva has arrived at a particular site, the decision to settle is influenced by the physical characteristics of the site (Suckling and Ioratti, 1996): larvae were more likely to build shelters and settle where two apple leaves or an apple fruit and a leaf were in physical contact. Plant volatile chemicals also may influence host selection: in small enclosed Petri dishes, neonate *E. postvittana* larvae were found in greater numbers in traps baited with apple fruit and leaf odors (Suckling and Ioratti, 1996). Based on these results, the authors suggested that neonate larvae may be attracted over short distances by volatile chemicals emitted by apple fruit and leaves.

After *E. postvittana* larvae have settled at a particular feeding site, they continue to gather information about the quality of the site. For example, on foods containing *Bacillus thuringiensis* endotoxins, larvae start feeding and begin to construct a silken shelter (Harris et al., 1997). A few hours later, these larvae stop feeding and abandon their shelters, and then walk for a period before again constructing a shelter. A similar sequence of events may occur on plants that appear to be good hosts on the basis of their surface features, but which are found to be unacceptable after plant tissue has been ingested.

In this paper we explore further the effects of plant chemicals on the behavior of *E. postvittana* neonate larvae. The following questions were addressed: Do neonate larvae exhibit directed responses to sources of volatile host plant chemicals? Do chemicals on the surface of host plants influence the behavior of neonate larvae as they walk over plant surfaces? Do host chemicals also influence how long larvae examine a particular site before abandoning it by spinning down on a silken thread? Are responses of larvae dependent on the dosage of host chemicals or the internal state of the larva during testing? Finally we obtained some preliminary details on the nature of these chemicals.

METHODS AND MATERIALS

Insects and Test Conditions. Eggs of *E. postvittana* were obtained from the laboratory colony at HortResearch, Auckland. In this colony, larvae were reared on a synthetic diet (Singh, 1974) at 20 ± 0.5°C. Adults were given a 10% honey solution for nourishment and wax paper as a substrate on which to lay eggs. Eggs were held in humidified plastic containers at 20 ± 2°C until sclerotization of the head capsule was apparent, and then were placed in a refrigerator at 5 ± 1°C. On days when experiments were conducted, eggs were removed from the refrigerator at 08:00 hr and held at 21 ± 1°C. Larvae began hatching about

30 min later and continued to hatch over the next few hours. Newly emerged larvae of this species are ~1.5 mm in body length and have a head capsule width of ~0.2 mm (Harris et al., 1995). Unhatched eggs were moved to a new plastic container every hour so that larvae of a particular age class could be used in experiments.

Larvae were generally tested within 1 hr after hatching but were never more than 4 hr old when tested (except in experiments on starvation). A larva hanging from a silk thread from the top or side of the plastic container was collected by touching a paint brush to the silk thread ~2–4 cm above the larva. The larva was then transferred to the experimental arena while suspended from the thread and gently placed on the release point (see experiments). Each larva was tested only once.

The behavioral responses of neonate larvae were tested in various arenas (see experiments), all of which were illuminated at 310 $\mu W/cm^2$ with Biolux fluorescent tubes with Quicktronic Deluxe ballasts (Biolux, Hamburg, Germany). All tests were run in a controlled environment room (21 ± 1°C, 60–70% relative humidity, 14L : 10D with lights turned on at 07:00 hr).

Apple Odors in the Wind Tunnel. Behavioral responses of neonate larvae to plant odors were tested in the 0.95-m-wide × 1-m-high × 2-m-long wind tunnel (Galanihe and Harris, 1997) located at the Massey University Plant Growth Laboratories, Palmerston North, New Zealand. A fan pushed air through the wind tunnel at speeds of 0.2–0.3 m/sec (Turbometer, Davis Instruments, Hayward, California). Odors were introduced into the wind tunnel by placing plant material in a chamber (0.95 cm wide × 1 m high × 15 cm long) upwind of the main body of the wind tunnel; the chamber was separated from the rest of the wind tunnel by two white cotton mesh screens. Materials placed in this upwind chamber were: (1) eight containers (6 × 4 × 4 cm) with moist soil placed on the floor of the chamber, (2) eight containers with moist soil with eight 60-cm-long branches of apple foliage (cultivar Braeburn, late January foliage) positioned vertically and spaced ca. 5 cm apart in the central zone of the chamber, and (3) eight containers with moist soil plus 20 apple fruits (5 cm diam. cultivar Braeburn, late January fruit), each fruit suspended (by a wire wrapped around its stem, which then was attached to a nylon fishing line) and placed in a 5 × 4 formation covering an area of 40–60 cm in the central zone of the wind tunnel. The apple cultivar Braeburn was chosen for this experiment because it was the only cultivar available from the Massey University Research Orchard (Palmerston North, New Zealand) that was not being heavily sprayed with insecticides in late January.

Larval responses to these odors were tested in the presence of blue or green color stimuli. Walking neonate *E. postvittana* larvae exhibit strong orientation responses to yellow–green reflectance but are inhibited when blue is added to green reflectance (Harris et al., 1995). Green or blue visual targets were posi-

tioned both upwind and downwind of the release point of the larva in the wind tunnel. The release point for a larva was the midpoint of a wooden rod (13 cm long × 2 mm diam.), the long axis of which was oriented parallel to the direction of airflow in the wind tunnel (also parallel to the floor of the wind tunnel), and which was suspended, by means of a clear nylon line at either end, at a height of 17.5 cm above the wind-tunnel floor. The upwind end of the wooden rod was 20 cm downwind of the white cotton mesh screen that stood at the upwind end of the wind tunnel. Two visual targets (both either blue or yellow–green) were positioned 10 cm upwind and 10 cm downwind of the ends of the wooden rod, so that an individual larva perceived similar visual stimuli when facing the upwind or downwind direction. Each target consisted of 12 evenly spaced (1.2 cm apart), vertically positioned blue or yellow–green colored strips of paper (1.5 × 29 cm) (see Harris et al., 1995, Fig. 1, for reflectance spectra). These strips of paper were supported on a 29- × 32-cm aluminum frame that stood perpendicular to the floor of the wind tunnel. The flat surface of the colored strips of paper was parallel to the screen at the upwind end of the tunnel. These visual targets did not appear to disrupt airflow significantly, the airflow being relatively smooth over the length of the wooden stick (airflow visualized with smoke). The wind tunnel floor was covered with a 2-cm layer of moist soil.

For each of the six combinations of odor (control, foliage, apple) and color stimuli (blue or yellow–green), five larvae were tested per block, with six blocks tested overall (30 larvae in total, randomized complete block design). Larvae were observed for 2 min and were scored "downwind" or "upwind" if they reached the downwind or upwind end of the stick, respectively, within 2 min. The small number of larvae (<3 per treatment) that did not walk to one of the two ends of the stick within 2 min were excluded from data analysis. A *G* test of independence was used to compare the effects of color and odor on upwind orientation (Sokal and Rohlf, 1981).

Larval Choice of Apple or Coprosma repens *Leaf Disks.* A choice test was conducted using apple (*Malus domestica*), a known host of *E. postvittana*, and *Coprosma repens*, a New Zealand plant species that has never been recorded as a host for *E. postvittana* larvae (Dugdale and Crosby, 1995). The arena for the choice test was a Petri dish (8.5 cm diam.) lined with damp filter paper (Whatman, Grade 1 Qualitative). In January, leaf disks (1 cm diam.) were made from fully expanded leaves cut from apple trees (cultivar Royal Gala) and *C. repens* trees located on the campus of the Mt Albert Research Centre, Auckland, New Zealand. Leaf disks were placed on the surface of the filter paper, abaxial side down, 5.5 cm apart from each other and 0.5 cm from the sides of the Petri dish. An individual larva was placed in the middle of the petri dish, halfway between the two leaf disks. Covers were placed on the dishes and Parafilm (American National Can, Chicago, Illinois) was used to seal the dishes to prevent the larva from escaping. Petri dishes set up in this manner were placed on plastic trays

(ten per tray) and covered with a light diffuser. Twenty-four hours later, Petri dishes were opened and the position of the larva and evidence of feeding and silk-spinning were recorded. Fifty dishes (replicates) were set up over two days using a completely randomized design. Distributions were analyzed using chi-square analyses (SAS Institute, 1995).

Extracts of Leaves and Fruit. Chemicals were extracted from the surface of fully expanded apple (cultivar Royal Gala) or *C. repens* leaves cut from trees growing on the campus of the Mt Albert Research Centre. Apple and *C. repens* leaves (of a similar size) were harvested from trees in January (within two days of the leaf disk choice test described above) and extracted within an hour of harvest. Three different extracts were made by dipping groups of four to five leaves (500 total) for 50 sec into 200 ml of one of three solvents: methanol (AR Grade, Riedel-de Haen AG, Seelze, Germany), dichloromethane (AR Grade, Mallinckrodt Specialty Chemical Co., Paris, Kentucky), or pentane (Nanograde, Mallinckrodt Specialty Chemical Co.) After leaves were extracted, the extract was evaporated from 200 to 100 ml (to 50 leaf equivalents/ml) by passive evaporation in a fume cupboard. Three extracts also were made of undamaged recently harvested apple fruit (cultivar Braeburn fruit from an organic orchard, harvested, and extracted in April) by dipping individual apples for 50 sec into methanol, dichloromethane, or pentane (40 apples/400 ml solvent). Fruit extracts were passively evaporated from 400 to 40 ml (1 fruit equivalent/ml). This extraction method probably extracted only chemicals present on the leaf or fruit surface (Eigenbrode and Espelie, 1995): all extracts were clear, plant material was not damaged, and the cut end of the petiole/stem did not contact the solvent. Extracts were stored at $-15°C$ until used.

Each extract was tested in a pairwise comparison with its solvent control (e.g., apple methanol extract vs. methanol). White filter paper disks (7 cm diam., Whatman Grade 2) were treated with either 5 leaf equivalents of an individual leaf extract (1 ml), 1 fruit equivalent (1 ml), or 1 ml of solvent. After the extract or solvent appeared to have evaporated, disks were held in an extractor hood for an additional 5 min. For the test, a single filter paper disk was placed horizontally on top of a 6-cm-diam. × 10-cm-high cylindrical platform that stood in the center of a cylindrical arena (30 cm diam. × 30 cm high) with walls of white construction paper and moist sand lining the bottom. An individual larva hanging on a silken thread was lowered onto the center point of the filter paper disk. The time the larva took to walk from the center to the edge of the filter paper disk was recorded using a stopwatch. All larvae reached the edge in less than 1000 sec. A complete block design was used, a block consisting of 10 larvae tested on a single solvent-treated filter paper disk, followed by 10 larvae tested on a single extract-treated filter paper disk. This procedure was repeated until a total of five blocks were completed over a five-day period (50 larvae tested for each treatment).

Because of limited availability of appropriate plant material (i.e., not sprayed with pesticides, not damaged by insects or diseases), the apple foliage and apple fruit used to make surface extracts were from different apple cultivars (Royal Gala and Braeburn, respectively). Hence, no statistical comparisons were made between the behavioral activities of apple foliage and apple fruit extracts. Instead, statistical comparisons were made within a single type of plant material, i.e., apple foliage, *C. repens*, or apple fruits, by comparing behavioral responses for each solvent–extract pair: pentane solvent–pentane extract, dichloromethane solvent–dichloromethane extract, and methanol solvent–methanol extract.

Behavioral Responses to Apple Leaf Extracts. To quantify some of the behavioral variables that contributed to larvae taking more time to walk from the center to the edge of filter paper disks, individual larvae were videotaped as they moved on disks treated with either dichloromethane or dichloromethane extracts of Royal Gala apple leaves (5 leaf equivalents). Filter paper disks were prepared and positioned as in the previous experiment, the only change being that smaller filter paper disks were used (5.5 vs. 7.0 cm diam.). A video camera (a black and white CCD camera, Panasonic model WV-BL200, Matsushita Electric Co. Ltd., Osaka, Japan) was mounted over an individual filter paper disk. A larva was placed in the center of the disk and its movement recorded until it contacted the edge of the disk. The treatment was then changed and another larva videotaped (complete block design with treatments paired over time). Twenty larvae were videotaped for each of the two treatments.

During playback of the videotape, the walking track of the larva was traced onto a grid. The position of the larva at 1-sec intervals was marked with ticks along the walking track, as were positions and times when the larva stopped forward movement (stops) and began walking again. The following data were collected for each larva: total time from the center of the disk to the edge (seconds), total distance travelled along the track (millimeters), number of stops while walking from the center to the edge, time allotted to stops vs. forward displacement, and velocity while moving (millimeters per second).

Spin-Down, Apple Leaf Extract, and Wind. The influence of the dichloromethane Royal Gala apple leaf extract on spin-down behavior was tested both in the presence and absence of wind, using a factorial design with four treatments: (1) solvent and no wind, (2) solvent and wind, (3) extract and no wind, and (4) extract and wind. For chemical treatments, 7-cm-diam. filter paper disks (Whatman Grade 2 Qualitative) were treated with either 5 leaf equivalents of the dichloromethane extract of apple leaves (1 ml) or dichloromethane (1 ml). For wind treatments, filter paper disks positioned as in previous experiments (i.e., treated side up, disk positioned at the top of a 0.5-cm-diam. cylindrical platform, 8 cm above a moist sand substrate) were held in a wind tunnel, 20 cm downwind of the cotton mesh screen at the upwind end of the wind tunnel described previously. The wind-tunnel fan was either set to produce a low wind-

speed (0.2–0.3 m/sec measured by a Turbometer, Davis Instruments, Hayward, California) or turned off so that there was virtually no horizontal air movement. In this experiment the cotton mesh screen at the upwind end of the wind tunnel was blue. This color choice was made because we wanted larvae to move away from the screen to the downwind edge of the filter paper disk, where it was easier to record the occurrence of spin-down. An individual larva, hanging on a silken thread, was placed on the center point of the disk. The time the larva took to walk from the center of the disk to the edge of the disk and the time the larva walked around the edge of the disk before spinning down on a silken thread were recorded.

Extract Dosage and Pretest Holding Times. Because behavioral responses of the larvae to leaf extracts were extremely variable (see section on Data Analysis), we explored the possibility of reducing variability by adjusting experimental procedures. Two tests were undertaken, increasing the dosage of the apple leaf extract and increasing the pretesting holding period of the larvae without food. In the first test, apple leaf extract was tested in the same manner described previously, using five dosages: 0, 2.5, 5, 10, or 15 leaf equivalents. In the second test, apple leaf extract was tested at 5 leaf equivalents with the three different holding periods, 0–1, 24–30, or 48–54 hr. For larvae tested at 0–1 hr, egg masses, from which neonate larvae were eclosing, were placed in a humidified plastic container and moved to another container on the hour, starting at 09 : 00 hr. For larvae held for 24–30 or 48–54 hr, newly eclosed individual larvae that had eclosed between 08 : 00 and 09 : 00 hr were kept in containers for 1 hr and then were placed individually in glass tubes (8 cm long, 1 cm diam.) stopped at either end with cotton. Glass tubes were kept in a humidified chamber to prevent dessication of the larvae. For each holding period, larvae were tested on both solvent-treated (dichloromethane) and extract-treated (5 leaf equivalents) 7.0-cm-diam. filter papers disks using the protocol described previously. Larvae were observed for a maximum of 600 sec. Larvae still walking on the disk at 600 sec were given a score of 600 sec. A complete block design was used, with the treatment changed after 5 larvae were tested per filter paper and a total of 10 blocks completed (50 larvae tested per treatment).

Rotary-Evaporated and Fractionated Leaf Extracts. To obtain some preliminary evidence on the nature of the behaviorally active chemicals in the dichloromethane extract of apple leaves, we recorded larval movement in response to an extract that had been rotary evaporated to dryness as well as an extract that had been fractionated. For the first test, a dichloromethane extract (180 leaf equivalents in 90 ml) of midseason apple leaves (cultivar Royal Gala) was divided into three equal parts (30 ml each). One part was used in an unaltered state to treat filter papers (original extract). The second part was evaporated to dryness in a rotary evaporator, then pumped under a slight vacuum (ca. 15 Kpa) at ambient temperature for an additional 15 min, and finally recon-

stituted in 30 ml of dichloromethane. The third part was evaporated to dryness in a rotary evaporator, then pumped under a slight vacuum (ca. 15 Kpa) at ambient temperature for an additional 12 hr, and finally reconstituted in 30 ml of dichloromethane. Each of the three extract treatments was applied at 5 leaf equivalents/2.5 ml to 7.0-cm-diam. filter papers and the same volume of solvent was used to treat control filter papers. The four treatments were tested using the methods described previously for testing leaf extracts, with 5 larvae/treatment/block and the eight blocks run on four separate days (total of 40 larvae tested per treatment, randomized complete block design).

For the fractionation, a 200-ml volume representing 630 leaf equivalents of the dichloromethane extract of midseason apple leaves was concentrated to 15 ml in a rotary evaporator under a slight vacuum and filtered. The filtered solids were washed with 20 ml of dichloromethane, which was added to the previous filtrate. Approximately 189 leaf equivalents of this filtrate were concentrated by rotary evaporation and added to an SPE column (International Sorbent Technology Ltd, Hengoed, UK; 5 g silica, 20 ml reservoir) which had previously been washed with 100 ml pentane. The following elution system was used to separate fractions: (1) 50 ml pentane, (2) 25 ml pentane/1% diethyl ether, (3) 25 ml pentane/5% diethyl ether, (4) 25 ml pentane/8% diethyl ether, (5) 25 ml pentane/15% diethyl ether, and (6) 35 ml diethyl ether. Each fraction was concentrated and stored at −15°C. Each of the six fractions was applied in a volume of 1.5 ml at 23 leaf equivalents/7.0-cm-diam. filter paper disk. The solvent control for this test was 1.5 ml of 50 : 50 pentane–diethyl ether per disk. These seven treatments were tested using the methods described previously, in a randomized complete block design, with 10 larvae/treatment in a block and six blocks run on six separate days (total of 60 larvae tested per treatment).

Data Analysis. The most commonly used behavioral variable measured, travel time from the center to the edge of a 7.0-cm-diam. filter paper disk, produced data sets that were not normally distributed (there were many outliers at both the bottom and top end of the response) and variances were heterogeneous. Elimination of outliers using normal quantile plots and transformation of data did not rectify these problems. Thus, these data were analyzed using nonparametric tests (SAS Institute, 1995): the Wilcoxon rank sum test when pairs of treatments were compared and the Kruskal-Wallis test when >2 treatments were compared. The null hypothesis for these tests is that the two groups have the same statistics of location, the median being a key location statistic (Sokal and Rohlf, 1981). For the Wilcoxon statistic, the magnitude of the rank sums determines the probability value of the test (Sokal and Rohlf, 1981). For the behavioral variables reported in Table 1 below, data sets met the assumptions of ANOVA (either before or after being transformed) and therefore were analyzed by two-way ANOVA (treatment and block effects) (SAS Institute, 1995). Block effects were not significant for any of the variables.

RESULTS

Apple Odors in the Wind Tunnel. When individual larvae were placed at the midpoint of a horizontally positioned wooden stick that was aligned parallel to the airflow in a wind tunnel, larvae typically remained at the point of release for 1–5 sec and then turned and walked downwind until they reached the end of the stick. Apple fruit or foliage odors had little or no effect on the percentage of larvae walking upwind (Figure 1). Larvae that were tested with yellow–green visual targets placed both upwind and downwind of the release point were more likely to walk upwind than larvae tested with blue visual targets, but still were more likely to walk downwind than upwind, regardless of odors (Figure 1). A test of independence indicated that the three variables, color × odor × walking direction were not jointly independent ($G = 17.06$, $df = 7$, $P < 0.05$). Additional G tests indicated that this overall lack of independence was due to the effects of color ($G = 14.62$, $df = 1$, $P < 0.01$) rather than odor ($G = 0.8$, $df = 2$, $P > 0.05$).

Larval Choice of Apple and C. repens *Leaf Disks.* Twenty-four hours after individual larvae were placed in small Petri dishes with two leaf disks, one of apple and one of *C. repens*, 45/50 larvae were found under the apple disk. All 45 larvae found on apple had constructed silken feeding shelters and had fed on the apple leaf disks, as evidenced by larval frass and holes in the leaf tissue.

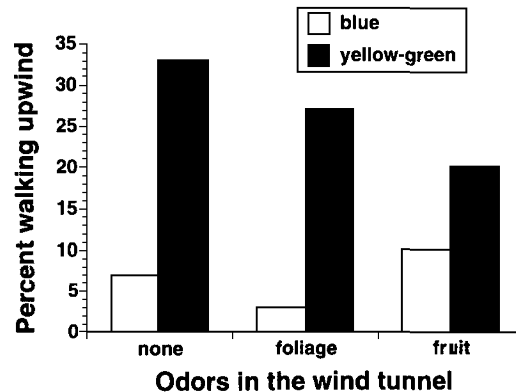

FIG. 1. Effects of color and apple odors on percentages of neonate larvae walking upwind in a wind tunnel. All other larvae walked in a downwind direction. Color screens, either both blue or both yellow–green, were placed upwind and downwind of the release point of the larva. Odor sources were placed behind a screen at the upwind end of the tunnel. Thirty larvae were tested for each of the six combinations of color and odor. Color ($P < 0.05$) but not odor ($P > 0.05$) increased percentages of larvae walking upwind (G test; 30 larvae tested/treatment).

Of the remaining five larvae, three were not found, one was dead on the Petri dish top, and one was found walking on the *C. repens* leaf disk. In the dish with the larva found walking on the *C. repens* disk, the apple disk showed signs of feeding but the *C. repens* disk did not. Inspection under magnification of all other *C. repens* disks failed to show any signs of silk spinning or feeding (i.e., frass). The position of larvae after 24 hr was not independent of leaf type (apple, *C. repens* other; $\chi^2 = 54.1$, $df = 1$, $P < 0.001$).

Extracts of Leaves and Fruit. When applied at 5 leaf equivalents to 7-cm-diam. filter paper disks, pentane, dichloromethane, and methanol extracts of apple leaves all elicited significant behavioral activity (Figure 2A). Of the three extracts, the dichloromethane extract produced the longest median travel times from the center to the edge of the disk, i.e., 468, 131, and 85 sec for the dichloromethane, pentane, and methanol extracts, respectively. As larval responses to a single extract-treated filter paper were tested over periods of up to 1–2 hr (i.e., 10 larvae tested per disk with response times of larvae averaging 8 min), we also used these data to investigate whether the first larva tested on a disk exhibited longer travel times than the tenth larvae that was tested on the (same) disk. Travel times of the first and tenth larva tested were not significantly different for pentane extract- or dichloromethane extract-treated disks ($P > 0.05$, Wilcoxon test) and travel times and order of testing (first, second, third, etc.) were not negatively correlated (pentane $F = 2.46$, $df = 1{,}48$, $P > 0.20$; dichloromethane $F = 0.63$, $df = 1{,}48$, $P > 0.50$); however, for the methanol extract, travel times were significantly longer ($P < 0.05$, Wilcoxon test) for the first larva tested (median 103 sec) than for the tenth larva tested (66 sec) and travel time was negatively correlated with order of testing of the larvae ($F = 7.14$, $df = 1{,}48$, $P < 0.01$).

When extracts of *C. repens* leaves were tested in the same manner at 5 leaf equivalents/disk (Figure 2B), none of the extracts was behaviorally active ($P > 0.05$, Wilcoxon test). When tested at 1 apple fruit equivalent/disk, pentane and methanol extracts of mature apple fruits were behaviorally active (Figure 2C, $P < 0.001$, Wilcoxon test). The dichloromethane extract of apple fruit was not behaviorally active ($P > 0.05$).

Behavioral Responses to Apple Leaf Extracts. Compared to larvae on a dichloromethane-treated disk, larvae on a dichloromethane apple leaf extract-treated disk took more than twice as much time to walk from the center to the edge of the disk (Table 1). Analysis of the walking paths and behavior of these larvae indicated that longer walking times on apple leaf extract-treated papers were related to several behavioral variables. First, larvae on extract-treated papers traveled longer distances after being released in the center of the disk and before reaching the edge (Table 1). Second, larvae exposed to extract walked at a slower net velocity (Table 1). Third, larvae on extract-treated disks stopped approximately four times more frequently than larvae on solvent-treated

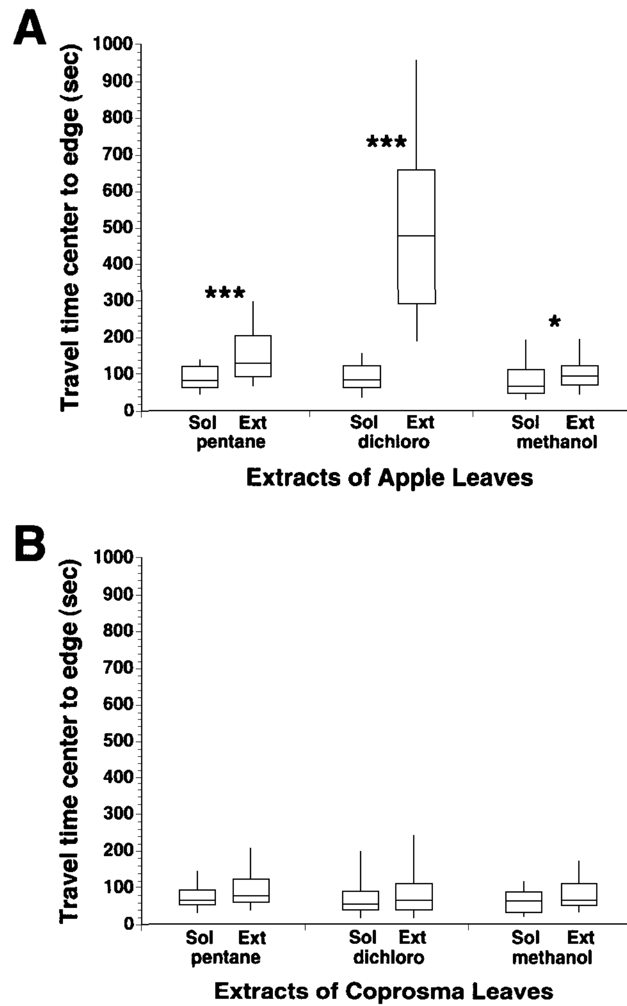

FIG. 2. Box plots of travel times taken by neonate larvae to walk from the center to the edge of filter papers treated with extracts (Ext) of (A) apple leaves (5 leaf equivalents/disk), (B) coprosma leaves (5 leaf equivalents/disk), or (C) mature apples (1 fruit equivalent/disk). Each extract was tested in a pairwise comparison with its solvent (Sol) with 50 larvae tested per extract or solvent. The top, middle, and bottom lines of the boxes correspond to the 75th, 50th (median), and 25th percentiles, respectively. The upper and lower vertical lines extend out to the 90th and 10th percentiles, respectively. Significant differences between solvent/extract pairs are indicated by *, $0.05 > P > 0.01$; **, $0.01 > P > 0.001$; and ***$P < 0.001$ (Wilcoxon sum rank test).

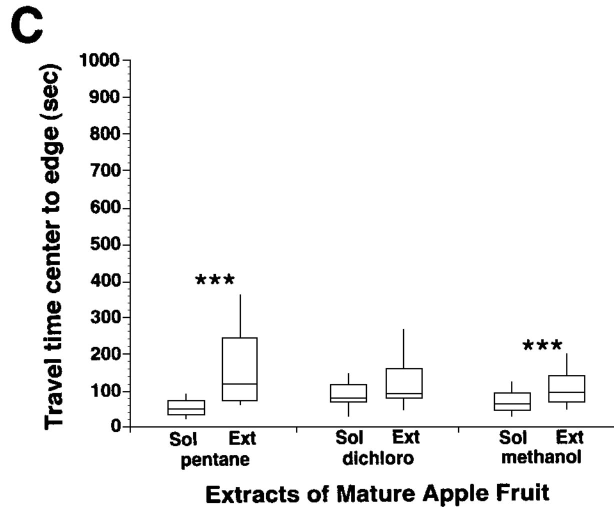

FIG. 2. CONTINUED.

disks (Table 1). The duration of stops on extract-treated papers was not significantly longer than on solvent-treated papers (Table 1). A multiple regression model exploring the contributions of these behavioral variables to travel times on both solvent- and extract-treated disks indicated that the number of stops, stop time, and walking velocity were good predictors of travel time (number of stops $F = 9.89$, $df = 1{,}35$, $P < 0.005$; stop time $F = 49.83$, $df = 1{,}35$, $P < 0.001$; velocity $F = 19.58$, $df = 1{,}35$, $P < 0.001$). Distance traveled was not a good predictor of travel time ($F = 0.50$, $df = 1{,}35$, $P > 0.50$).

Spin-Down, Apple Extract, and Wind. An experiment measuring the effects of dichloromethane apple leaf extract and wind on walking times and spin-down indicated that the extract influenced both behavioral variables (Figure 3). Travel times from center to edge were significantly different among the four treatments ($\chi^2 = 99.13$, $df = 3$, $P < 0.001$, Kruskal-Wallis test) and were slower on extract-treated disks than on solvent-treated disks both in no wind and wind conditions (Figure 3A). As the screen at the upwind end of the wind tunnel was blue, a color that repels *E. postvittana* larvae (Harris et al., 1995), all larvae walked towards the downwind end of the wind tunnel. In the case of extract-treated disks, larvae reached the downwind edge of the disk significantly faster in wind conditions (median 102 sec) than in no-wind conditions (median 133 sec; $P < 0.005$, Wilcoxon test). Thus, it appeared that walking velocity over extract-treated surfaces was faster when larvae walked in the presence of a wind that was moving in the same direction that they themselves were walking. In the case of solvent-

TABLE 1. BEHAVIOR OF NEONATE *E. postvittana* LARVAE MOVING FROM CENTER TO EDGE OF 5.5-CM-DIAMETER FILTER PAPER DISK TREATED WITH DICHLOROMETHANE (SOLVENT) OR 5 LEAF EQUIVALENTS OF DICHLOROMETHANE EXTRACT OF APPLE LEAVES

	Mean ± SE				
Chemicals applied to filter paper disks	Travel time from disk center to edge (sec)	Distance traveled (mm)	Walking velocity (mm/sec)	Number of stops from center to edge	Mean duration of stops (sec)
Solvent	33 ± 10	31 ± 2	1.23 ± 0.07	1.6 ± 0.9	2.8 ± 0.5
Apple leaf extract	74 ± 10	37 ± 2	0.93 ± 0.07	6.2 ± 0.9	3.5 ± 0.5
ANOVA	$P < 0.005$[a]	$P < 0.05$	$P < 0.005$	$P < 0.001$[a]	$P > 0.05$

[a]These variables were log-transformed before being subjected to ANOVA. Block effects were not significant.

treated disks, larvae in no-wind conditions and larvae in plus-wind conditions had similar travel times to the downwind edge of the disk.

The time at which larvae initiated spin-down after reaching the edge of the disk was influenced by wind conditions as well as the dichloromethane apple leaf extract (Figure 3B). In wind conditions, larvae delayed spin-down on extract-treated disks relative to solvent-treated disks ($P < 0.001$, Wilcoxon test), but in no wind conditions, larvae had similar spin-down times on the two treatments ($P > 0.05$, Wilcoxon test). Overall, larvae tested in wind conditions initiated spin-down from the edge of the disk sooner (median of 146 sec for solvent + extract larvae combined) than larvae tested in no wind conditions (median of 270 sec for solvent + extract larvae combined) (Wilcoxon test, $P < 0.001$). For Travel times from the center to the edge and times from the edge to first spin down were not correlated ($P > 0.05$).

Extract Dosage and Pretest Holding Time. Increasing dosage of the dichloromethane apple leaf extract did not rectify problems with data that disallowed analysis by parametric statistics, i.e., just as with data at 5 leaf equivalents, data at 10 or 15 leaf equivalents were not normally distributed and had heterogenous variances. Transformation of data and elimination of outliers still did not significantly improve these problems. Nonparametric tests revealed that center to edge travel times were significantly different across the five treatments ($\chi^2 = 26.02$, $df = 4$, $P < 0.001$, Kruskal-Wallis test). Relative to solvent-treated disks (median travel time of 64 sec), disks treated with 5, 10, or 15 leaf equivalents (median travel times of 121, 109 and 143 sec, respectively) were behaviorally active, but disks treated with 2.5 leaf equivalents were not (median travel time of 78 sec). However, P values for Wilcoxon tests comparing travel times for solvent- and extract-treated disks control did not increase with increasing dosage,

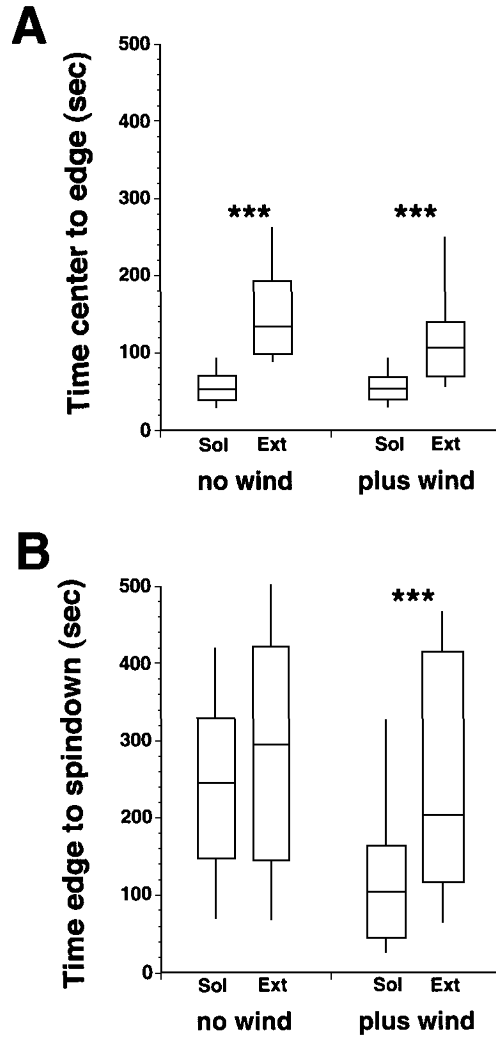

FIG. 3. The influence of apple leaf extract and wind on (A) center to edge travel times and (B) spin-down times of neonate larvae. Filter paper disks were treated with dichloromethane (Sol) or the dichloromethane apple leaf extract (Ext) at 5 leaf equivalents/disk. See Figure 1 for explanation of box plots. For no-wind or plus-wind conditions, significant differences among solvent/extract pairs are indicated by $^{*}0.05 > P > 0.01$; $^{**}0.01 > P > 0.001$; $^{***}P < 0.001$ (Wilcoxon test). Fifty larvae were tested for each of the four treatments.

i.e., the statistical difference in travel times on solvent- and extract-treated disks was not greater for 15 LE than for 5 LE or 10 LE.

When newly eclosed larvae were held without food for different periods of time before being tested (0–1, 24–30, or 48–54 hr), larvae at all holding periods showed significantly longer center-to-edge travel times on dichloromethane apple leaf extract-treated disks than on solvent-treated disks ($P < 0.001$, Wilcoxon test). However, holding larvae for longer periods before testing did not improve data (i.e., did not create normal distributions and homogeneous variances), did not increase P values for tests comparing solvent and extract, and significantly increased the time needed to test both extracts and solvents. When holding time was increased from 0–1 hr to 48–54 hr, median travel times on solvent-treated disks increased significantly from 53 to 130 sec ($P < 0.001$, Wilcoxon test) and on extract-treated disks increased from 167 to 203 sec ($P < 0.05$, Wilcoxon test).

Rotary-Evaporated and Fractionated Leaf Extracts. When tested on dichloromethane apple leaf extracts that had been evaporated to dryness in a rotary evaporator and then either evaporated for a further 15 min or a further 12 hr, larvae exhibited similar center-to-edge traveling times on the two extracts (Figure 4). When data for these two evaporated extracts were combined, travel times on the evaporated extracts were longer than on the solvent control ($P < 0.001$, Wilcoxon test) but shorter than on the control dichloromethane apple

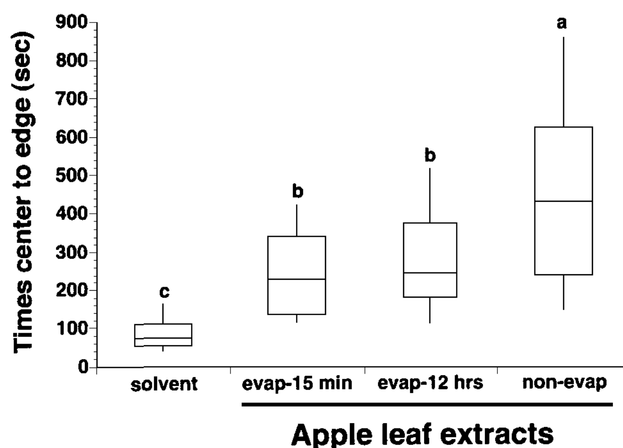

FIG. 4. Behavioral effects of reducing volatile components in the dichloromethane extract of apple leaves. Extracts were either not evaporated (non-evap) or rotary evaporated to dryness and then evaporated for an additional 15 min (evap-15 min) or 12 hours (evap-12 hrs). All three extracts were tested at 5 leaf equivalents/disk. Travel times accompanied by different letters are significantly different at $P < 0.001$ (Wilcoxon test).

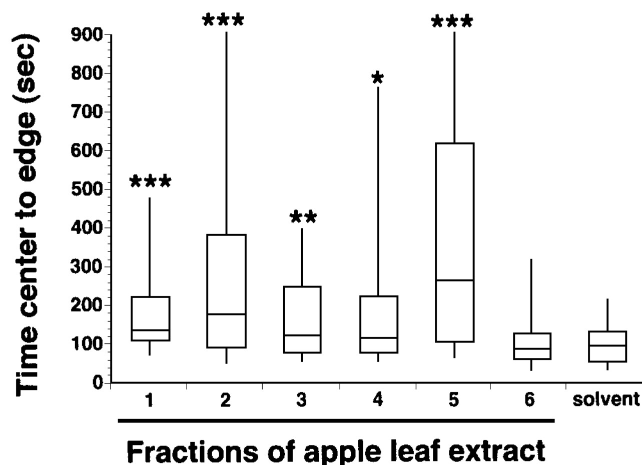

FIG. 5. Behavioral activity of six fractions of the dichloromethane extract of apple leaves, tested at 23 leaf equivalents/disk. The solvent was a 50 : 50 mixture of pentane and diethyl ether, the two solvents used for fractionation. Each fraction was compared to the solvent control using the Wilcoxon test (*, $0.05 > P > 0.01$; **$0.01 > P > 0.001$; and ***, $P < 0.001$).

leaf extract ($P < 0.001$, Wilcoxon test). Travel times on the four treatments were significantly different ($\chi^2 = 73.78$, $df = 3$, $P < 0.001$, Kruskal-Wallis test).

When a dichloromethane apple leaf extract was fractionated and the six fractions were tested relative to a pentane–ether control (50 : 50 by volume), there were significant differences in travel times across the seven treatments ($\chi^2 = 58.25$, $df = 6$, $P < 0.001$, Kruskal-Wallis test). Relative to the solvent-treated control, larvae exhibited significantly longer walking times on five out of six of the fractions (Figure 5), with the median travel time longest on fraction five.

DISCUSSION

In our wind-tunnel experiment, regardless of the presence of apple leaf or fruit odors, most neonate *E. postvittana* larvae turned downwind within seconds of their introduction into the wind tunnel and proceeded to walk, without stopping, to the downwind end of the stick (Figure 1). Color stimuli, tested in conjunction with odors, modified these responses somewhat: more larvae turned upwind when yellow–green (10/30) rather than blue (2/30) visual targets were placed both upwind and downwind of the release point. Our interpretation of these results is that on a surface that itself is not a potential feeding site, the direction in which *E. postvittana* larvae walk is influenced by both visual stimuli and

wind direction. If no stimulatory visual cues are detected (i.e., no yellow–green reflectance; Harris et al., 1995), the larva turns its head away from the wind and walks downwind. However, if during the assessment of visual stimuli and wind direction, stimulatory color cues are detected in the upwind direction, some larvae will choose to walk upwind. Perhaps for these larvae, the stimulatory effect of the upwind visual target outweighs the inhibitory effects of walking into the wind.

While neonate *E. postvittana* larvae did not respond to sources of volatile apple chemicals by walking upwind, they did respond to apple chemicals that were present on walking surfaces. In an assay in which individuals were placed in the middle of a 7-cm-diam. filter paper disk and scored for the time they took to travel from the center to the edge of the disk, larvae traveled more slowly across disks treated with extracts of apple leaves (Figure 2A) or apple fruit (Figure 2C). Extracts of *C. repens*, a plant that was rejected by all *E. postvittana* larvae in favor of apple in a choice assay, had no apparent effect on travel times (Figure 2B). These results imply that *E. postvittana* larvae, while walking, can distinguish between host and nonhost leaves (at least in the case of these two plants) on the basis of chemicals present on, or emitted by, their surfaces. Such an ability to make an initial assessment of host quality while walking over plant surfaces would be of obvious benefit to *E. postvittana*, allowing neonate larvae to minimize the time spent examining plants that do not appear to be suitable hosts while maximizing the time spent on plants that deserve further examination.

As larvae on *C. repens* extract-treated disks did not walk faster than larvae on disks treated with the respective solvents used to make these extracts, it is tempting to speculate that apple leaves and *C. repens* leaves differ only in the amounts of stimulatory chemicals they contain. However, caution must be taken in overinterpreting the results of behavioral assays with crude extracts. For example, there may be both stimulants and inhibitors (i.e., chemicals that elicit travel times that are slower and faster than those on solvent controls, respectively) in the crude extract of *C. repens*, the combination of which results in travel times that look very much like those on solvent-treated disks.

It will be of interest to determine whether behaviorally active chemicals are present on the surfaces of the other plant hosts of *E. postvittana* (more than 250 species). At this point in our studies we cannot even generalize about the presence of behaviorally active chemicals on apples: only a single apple cultivar was used to produce our leaf extract (Royal Gala) and a different single apple cultivar was used to produce our fruit extract (Braeburn). We intend to characterize, at different stages of apple leaf/fruit development, the behaviorally active chemicals on different cultivars, as well as any behavioral preferences larvae might have for settling and feeding on leaves or fruit. Such studies will provide information about the influence of plant surface chemistry on the seasonal distribution of larvae within and between apple trees. Additional studies will examine

the chemistry of other plant species that serve as hosts for *E. postvittana* larvae. Do these species also have behaviorally active chemicals on their surfaces and are these chemicals similar to those found on apple?

After establishing the behavioral activity of apple extracts, two experiments were run to determine whether differences between travel times on solvent-treated disks and extract-treated disks could be increased by changing one of two experimental procedures: (1) increasing the dosage of the extract or (2) holding larvae for longer periods without food before testing. Neither change in experimental procedure significantly increased differences between travel times on solvent- and extract-treated disks. Indeed, holding larvae for 48–54 hr before testing may have reduced the degree to which larval behavior differed on solvent- versus extract-treated disks. This was because, relative to larvae held for 0–1 hr before testing, larvae held for 48–54 hr not only had longer travel times on extract-treated disks (median travel time increased from 167 sec for 0–1 hr larvae to 203 sec for 48–54 hr larvae) but also had longer travel times on solvent-treated disks (median travel times increased from 53 sec for 0–1 hr larvae to 130 sec for 48–54 hr larvae). As neonate *E. postvittana* larvae only live for approximately 72 hr when held without food (Harris, unpublished results), larvae held without food for 48–54 hr before testing may have had longer travel times because they walked more slowly (i.e., general debilitation), or because, like starved locusts (Williams, 1954; Sinoir, 1969), they stopped more frequently and during these stops bit any available surface, regardless of its chemical characteristics.

Scoring travel times of *E. postvittana* larvae was a convenient way to establish the presence of behaviorally active chemicals in extracts but revealed little about the actual behavioral effects of these chemicals. Analysis of video-records of larvae walking from the center to the edge of extract- and solvent-treated disks provided a clearer picture of these behavioral effects. In the case of the dichloromethane extract of apple leaves, dichloromethane being a solvent that extracts epicuticular lipids from the plant surface (Eigenborde and Espelie, 1995), larvae on extract-treated disks walked more slowly and traveled longer distances (Table 1). The significantly longer center-to-edge traveling distances imply that host-plant chemicals modify the walking behavior of larvae, causing them to take more circuitous routes in contrast to the relatively straight route taken by larvae walking on solvent-treated disks (the mean center-to-edge travel distance of control larvae was 31 mm across a filter paper disk that had a radial distance of 27.5 mm). In addition to slower walking speeds and more circuitous walking paths, larvae walking on extract-treated disks stopped about four times more frequently than larvae on solvent-treated disks (Table 1). In the diamondback moth, *Plutella xylostella* (L.), neonate larvae exhibit similar responses (i.e., slower walking speed, increased stopping) to the epicuticular lipid chemical components of susceptible (nonglossy) but not resistant (glossy) genotypes of cabbage (Eigenbrode et al., 1991).

Further observations of *E. postvittana* neonate larvae under a microscope (Harris, unpublished observations) have revealed that during stops on treated filter paper, the position of the head is changed so that the mouthparts, which project forward when the neonate larva walks, now project downwards. Accompanying this change in head position are movements of the mandibles that look like biting. Furthermore, mouthpart structures posterior to the mandibles, i.e., the maxillary palps and the galea, are brought in closer contact with the filter paper. Caterpillars have numerous gustatory and olfactory sensilla on the tip of the maxillary palps and galea (Grimes and Neunzig, 1986a,b). If *E. postvittana* larvae were indeed engaging additional sensory systems and/or biting extract-treated surfaces, it may have been at this point that our filter paper models no longer mimicked the types of chemical stimuli that would normally be encountered when examining a real leaf. Indeed, on our filter paper models, the lack of these additional plant constituents may have been the reason that durations of stops on extract- and solvent-treated papers did not differ significantly (Table 1).

Another behavior that is influenced by leaf surface chemicals is spin-down on a silken thread. Thus, after walking from the center to the downwind edge of a disk held in a wind tunnel at windspeeds of 0.2–0.3 m/sec, larvae on dichloromethane apple leaf extract-treated disks delayed spinning down relative to larvae on solvent-treated disks (Fig. 3B). Interestingly, when there was minimal air movement in the wind tunnel, spin-down was delayed regardless of whether the disk was treated with extract or solvent (Fig. 3B). These results suggest that when larvae reach the edge of whatever surface they have been walking on, the decision to spin-down is based on information gathered by at least two different sensory systems: mechanoreceptors that provide information about air movement and chemoreceptors that provide information about plant chemicals. Thus, at the edge of a surface, the detection of light winds by larvae triggers spin-down. Leaf surface chemicals counter this effect of wind by stimulating larvae to examine the surface for longer periods before abandoning it by spinning down.

Tests comparing travel times of *E. postvittana* on fractions of the extract or evaporated extracts provided some preliminary information on the nature of the behaviorally active chemicals in the most active of the apple foliage extracts, the dichloromethane extract. First, there appear to be at least two chemicals in the extract responsible for its behavioral activity (Figure 5). These chemicals range from little or no polarity (in fraction 1) to relatively polar chemicals (in fraction 5). Second, the dichloromethane apple leaf extract appears to contain both volatile and involatile components that influence the behavior of *E. postvittana* larvae. Evidence for this can be seen in Figure 4. Travel times of larvae decreased when the volatile components of the extract were removed by rotary evaporation under a slight vacuum (ca. 15 Kpa) for 15 min or 12 hr. However, after these volatile chemicals were removed, the extract was still behaviorally active

enough to produce travel times that were significantly longer than those on the solvent control. Thus, after rotary evaporation, involatile chemicals remained in the extract and were behaviorally active.

A sense of how volatile some of these chemicals might be is provided by two further observations. First, when the dichloromethane extract of apple leaves was tested (Figure 2), each treated filter paper was used to test 10 larvae, each of which took an average of 8 min to travel from center to edge. As the first larva tested did not exhibit longer travel times than the tenth larva, which was tested approximately 1–2 hr later, it would appear that the behaviorally active volatile components of the extract are not highly volatile, i.e., they do not evaporate soon after placement on the filter paper. The moderate volatility of the components is further supported by the fact that 15 min of pumping under a slight vacuum was sufficient to remove this chemical(s): pumping under slight vacuum for a further 12 hr caused no further decrease in behavioral activity (Figure 4).

That volatile chemicals from apple leaves and fruit influence the behavior of neonate *E. postvittana* larvae is supported by the studies of Suckling and Ioratti (1996) who found more larvae in traps baited with sources of volatile chemicals from apple leaves and fruit. However, it is still unclear what behaviors of *E. postvittana* are triggered by volatile plant chemicals. As in other insect herbivores, volatile plant chemicals could be important in host-finding from a distance (chemotaxis or chemically mediated anemotaxis) (Visser, 1986; Bell, 1991), working over shorter distances, at higher concentrations, or at different windspeeds than those in our wind-tunnel experiment (Figure 1). On the other hand, *E. postvittana* spend most of their time as neonate larvae within the boundary layer of air above the plant surface, a location where volatile plant chemicals are often in high concentrations. Thus it is possible that the sole effect of volatile plant chemicals is to regulate examining and feeding behavior when the larva is already in contact with the plant (Chapman, 1995).

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INSECT-INDUCED SYNTHESIS OF PHYTOECDYSTEROIDS IN SPINACH, *Spinacia oleracea*

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Abstract—Spinach (*Spinacia oleracea*) foliage is known to synthesize and accumulate insect molting hormones, predominantly in the form of 20-hydroxyecdysone (20E). We previously demonstrated that root 20E accumulation is increased following root damage. We designed two further experiments to address root responses to both mechanical and insect damage. In plants grown hydroponically, removal of 35% or less of the root mass did not result in changes in root 20E levels. However, removal of 70% of the root mass stimulated 6.0- and 1.5-fold increases in the root and shoot 20E concentrations, respectively. The effects of insect damage on soil-grown plants were investigated by infesting plant roots with black vine weevil (BVW: *Otiorhynchus sulcatus*) larvae and allowing them to feed for seven days. Decreases in root mass occurred in young plants; however, no changes were detected in mature plants. In all cases, root herbivory resulted in at least a 3.0-fold increase in root 20E concentrations. Our previous experiments implicated jasmonic acid and the analog methyl jasmonate (MJ) in signaling the damage-induced accumulation of root 20E levels. We investigated the activity of other phytohormones and growth regulators (GRs) on the 20E accumulation patterns of young plants as a means of examining the significance of jasmonates in the induction response. Hydroponic additions of MJ (0.5 μM) and the synthetic auxin, 1-naphthaleneacetic acid (NAA; 0.5 μM), resulted in significant increases in root 20E levels. At the concentrations

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tested, indole-3-acetic acid (IAA), gibberellic acid (GA_3), abscisic acid (ABA), and *trans*-zeatin (Z) had no effects on root 20E concentrations. However, both NAA (0.5–5.0 μM) and Z (5.0 μM) treatments caused increases in the root/shoot dry mass ratios, indicating shifts in resource allocation to the roots. Treatments involving ABA (5.0 μM) and Z (0.5–5.0 μM) caused significant increases in shoot 20E concentrations. No other hormone treatments altered shoot accumulation patterns. The mechanisms underlying the root 20E induction phenomena were investigated through the incorporation of [2-^{14}C]mevalonic acid ([^{14}C]MVA). Within one day, excised roots readily incorporated radioactivity into 20E from [^{14}C]MVA. In intact plants, [^{14}C]MVA absorbed by the roots was rapidly incorporated into root 20E pools following damage and MJ treatments. This implies that the wound-induced root 20E accumulation is the result of increased *de novo* 20E synthesis in the root.

Key Words—Spinach, *Spinacia oleracea*, induced defense, damage, 20-hydroxyecdysone, phytoecdysteroid, methyl jasmonate, jasmonic acid, root herbivory, *Otiorhynchus sulcatus*, abscisic acid, gibberellic acid, indole-3-acetic acid, 1-naphthaleneacetic acid, *trans*-zeatin, root–shoot ratio.

INTRODUCTION

Proposed functions for phytoecdysteroids (PEs) in plants can be divided into two major categories. First, PEs may perform a direct physiological function. This includes hormonal, developmental, and sterol transport hypotheses (Heftmann, 1975; Sláma, 1979; Machácková et al., 1995). Second, PEs may protect plants against phytophagous insects by acting as feeding deterrents (Ma, 1969; Tanaka et al., 1994) or by disrupting normal development (for reviews see Bergamasco and Horn, 1983; Lafont et al., 1991; Camps, 1991). However, these two functions may not be mutually exclusive (Berenbaum, 1995), especially considering that none of the proposed roles for PEs have been unequivocally demonstrated. An increased understanding of PE regulation in plants, including the identification of relevant stresses and signals that alter accumulation patterns, will aid in constructing testable hypotheses regarding these putative functions.

Increases in many plant defense chemistries are induced by insect herbivory (Karban and Baldwin, 1997). These changes are often signaled by plant derived wound signals that initiate from the site of damage (Pearce et al., 1991; Davis et al., 1991; Baldwin et al., 1997). However, mechanical damage treatments involving tissue removal alone do not always mimic responses produced by insect herbivory (Baldwin, 1990; Wildon et al., 1992). In addition to the physiological changes following mechanical damage, plants, may produce specific responses to insect attack (Alborn et al., 1997). Thus, induced plant responses following mechanical damage should only be equated to those caused by actual insect herbivory after empirical testing. Recently, we described the rapid phytochemi-

cal induction of 20E in the roots of spinach following damage (Schmelz et al., 1998). Our first objective was to determine the effects of root removal on the 20E induction response in the absence of insect-derived variables. The second was to establish whether the root induction response occurs in soil grown plants following insect herbivory.

Investigations of induced plant responses, and their significance, can be aided by uncoupling phytochemical induction from physical changes caused by damage (Thaler et al., 1996; Stout et al., 1998; Baldwin, 1998). The ability to induce a wide range of 20E levels, in the absence of damage, would enable the putative protective roles of PEs to be examined. A prerequisite to achieving this goal is a general understanding of the plant signal transduction pathways leading to PE induction. We previously demonstrated the effects of jasmonates and octadecanoic acid pathway inhibitors on 20E accumulation (Schmelz et al., 1998). However, little is known about the effects of other phytohormones and growth regulators (GRs) on PE accumulation. By using callus cultures of *Trianthema portulacastrum*, Ravishankar and Mehta (1979) demonstrated that low concentrations (0.2–2.0 ppm) of 1-naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) stimulated PE accumulation, while indole-3-acetic acid (IAA) had no effect. Gibberellic acid (GA_3) possessed only weak PE-inducing activity, whereas a combination of 2,4-D and kinetin promoted the maximal PE production. Currently, many cell and tissue culture studies include the synthetic auxins NAA and 2,4-D in the media to promote PE synthesis (Camps et al., 1990; Corio-Costet et al., 1993; Tomás et al., 1993); however, the impact of either natural or synthetic auxins on PE synthesis in intact plant systems has not been described. Thus, our third objective was to compare the relative 20E-inducing activity of methyl jasmonate (MJ) to other phytohormones and GRs.

Mature spinach leaves are known to be active sites of 20E biosynthesis. In contrast, young apical leaves are not biosynthetically active; however, they do accumulate high levels of 20E through transport from other tissues (Grebenok and Adler, 1991, 1993). While dynamic transport processes appear to occur in the leaves, the interactions between root and shoot 20E pools remain unknown. Damage-induced increases in root 20E could be caused by basipetal transport of shoot 20E to the roots or by increases in de novo biosynthesis in the roots. Induction could also occur through the release of free 20E from unmeasured conjugated pools (Grebenok et al., 1994). Our fourth objective was to examine the role of root PE biosynthesis in the production of induced increases in root 20E. Knowledge of the mechanism of induction would increase our understanding of how spinach roots and phytophagous insects interact and eventually aid in manipulating these interactions.

Given these goals, we addressed the following questions: (1) Does root tissue removal alone cause increases in root 20E levels? (2) In soil grown plants, does damage caused by obligate root herbivores result in a root 20E induction?

(3) Aside from MJ, do other phytohormones and GRs have significant effects on 20E accumulation patterns? (4) What is the physiological mechanism behind damage induced accumulation of root 20E?

METHODS AND MATERIALS

Chemicals. Gibberellic acid (GA_3), (±)-*cis,trans*-abscisic acid (ABA), indole-3-acetic acid (IAA), 1-naphthaleneacetic acid (NAA), *trans*-zeatin (Z), methyl jasmonate (MJ), and all plant nutrient components were purchased from the Sigma Chemical Co. (St. Louis, Missouri). The *RS*-[2-^{14}C]mevalonic acid DBED salt ([^{14}C]MVA) was obtained through NEN Life Science Products (Boston, Massachusetts). HPLC-grade solvents were acquired from the Fisher Chemical Company (Pittsburgh, Pennsylvania).

Plant Growth. Spinach (*Spinacia oleracea* var. Avon) plants were grown in an environmental chamber at 23°C, with a 12L : 12D cycle and 35% humidity. Fluorescent lighting supplied approximately 145 μmol photons/m^2/sec to the leaf surface. Seeds, acquired from W. Atlee Burpee Co. (Warminster, Pennsylvania), were germinated in vermiculite and allowed to grow for approximately 10 days. Seedlings at the cotyledon stage were transplanted into individual opaque 1-liter plastic cups and grown hydroponically for an additional 10–20 days. The hydroponic nutrient solution used follows exactly from Schmelz et al. (1998). One day prior to each experiment, plants of uniform morphology and size were selected and weighed. Treatment groups having similar mean fresh masses were formed by sorting plants based upon fresh mass and assigning them to groups by consecutive divisions (see Ohnmeiss and Baldwin, 1994). At the start of each experiment, plants were randomly assigned growth-chamber shelf positions. No additional nutrients were added to the hydroponic solutions before or during the experiments; the solution levels were maintained by the addition of distilled water as needed.

Plants required for soil grown experiments were transferred from seedling flats 10 days after germination to clear 1-liter cups with approximately 300 ml of soil adjusted to pH 7 with aqueous KOH. The soil consisted of two thirds vermiculite (Therm-o-rock Industries, Inc., Chandler, Arizona) and one third Brazito sandy loam (74.7% sand, 14.6% silt, 10.7% clay, 0.53% organic carbon). The field-collected sandy loam was first autoclaved to remove potential pathogens. All plants initially received a 1-liter nutrient equivalent (see Schmelz et al., 1998) of the standard hydroponic solution. This nutrient dose was administered again after 14 days of growth. Germination dates were staggered such that young and mature plants (35 and 55 day old plants, respectively) were available simultaneously. All plant containers had hundreds of pinholes on the undersides that enabled bottom watering through capillary action.

Phytoecdysteroid Quantification. After the treatment and response period (three to seven days), plants were dissected into roots and shoots, then frozen and lyophilized to dryness. Each sample was ground to a fine powder by using a Wiley mill (850 μm mesh; Thomas Scientific), and a weighed portion, (normally 50–60 mg) was extracted in 10 ml of methanol for 48 hr. A 7-ml aliquot of this extract was mixed with 3 ml H_2O and partitioned against 10 ml hexane. After clear-phase separation, 8 ml of the aqueous methanol layer was removed and evaporated to dryness. The residue was resolubilized in 5 ml H_2O and partitioned against 5 ml of butanol. A 4-ml aliquot of the butanol phase was evaporated and resuspended in 400 μl of methanol prior to analysis. For quantitative purposes, all partitions utilized previously countersaturated solvents. Reverse-phase high-performance liquid chromatography (RP-HPLC) of spinach PEs was carried out on a C_{18} Alltech Spherisorb ODS-2, 4.6-mm × 150-mm, 5-μm particle column. An isocratic 2-propanol–H_2O (12 : 88 v/v) mobile phase was used that separates 20E from polypodine B (Morgan and Marco, 1990; Schmelz et al., 1998). For each experiment, 20E quantification was extrapolated from concurrently generated external standard curves, constructed from independent weighings of 20E.

[^{14}C]*Mevalonic Acid-Treated Tissues.* With the necessary precautions, ^{14}C-radiolabeled samples were treated similarly to the nonradiolabeled samples. Following lyophilization, individual samples were pulverized with a glass rod inside 20-ml scintillation vials. A weighed portion (50–60 mg) was extracted in 10 ml methanol for 48 hr. An aliquot (7 ml) was removed and mixed with 3 ml H_2O and 10 ml hexane. The hexane layer was removed, saved, and replaced with a new aliquot of hexane. One day after this second phase separation, the entire 10 ml of the methanol/H_2O layer was removed and evaporated to dryness. The residue was partitioned between 5 ml butanol and 5 ml H_2O. One day after separation, 4.5 ml of the butanol layer was removed, evaporated to dryness, resolubilized in a minimal volume of methanol, and analyzed by HPLC. The isolated 20E peak was quantified as described, and the corresponding fraction was collected. The determination of ^{14}C incorporation into 20E follows from Grebenok and Adler (1991) and utilized a Beckman LS-1801 liquid scintillation counter with Bio-Safe II counting cocktail (Research Products International Corp, Mt. Prospect, IL). All values of ^{14}C incorporation into 20E are reported in counts per minute, from which the average background fluorescence of the mobile phase was subtracted.

Plant Response to Root Excision. We created a highly reproducible root damage treatment in hydroponically grown plants to contrast root herbivory in soil grown plants. Thirty-six plants were divided among six groups ($N = 6$, 3.99 ± 0.14 g). Three groups involved temporarily removing the plants from the hydroponic solution, straightening out the roots, and excising with a razor either 30%, 60%, or 90% of the total root length starting from the most distal tip. Plants were returned to their original solution and allowed to grow during the four-day

treatment and response period. Untreated controls were harvested at day 0 and day 4. A MJ (0.45 μM)-treated positive control was included as a test for 20E inducibility (Schmelz et al., 1998). On day 0, the lengths of excision resulted in an average 15%, 35%, and 70% root mass removal, respectively.

Root Herbivory by Black Vine Weevil Larvae. Investigations of root 20E induction in spinach have thus far been limited to herbivory simulation experiments with hydroponically grown plants. This raises two primary questions. First, are soil-grown roots actually in the uninduced state or does root abrasion during soil-based growth naturally produce induced roots? Second, is the 20E induction response following root mechanical damage in hydroponic plants similar to the response produced by insect herbivory in soil grown plants? To address these questions, we selected a highly polyphagous obligate root herbivore that would not be affected by root PEs. Black vine weevil (BVW: *Otiorhynchus sulcatus*) larvae are known to feed on the roots of over 100 species of plants, with preferred hosts ranging from strawberry (*Fragaria* spp.) to yew trees (*Taxus* spp.) (Moorhouse et al., 1992). These insects were assumed to be resistant to dietary PEs because members of the genus *Taxus* are known to contain high levels of both ponasterone A and 20E (Ripa et al., 1990). Plants of two different age classes were selected for comparative purposes because the inducibility of plant defense compounds often changes over ontogeny (Ohnmeiss, 1998). At the beginning of the experiment, plants were either 35 or 55 days old. They are subsequently referred to as young and mature plants, respectively. Immediately prior to the start of the experiment, the soil-grown plants were visually paired based on size, morphology, and age. From each pair, one experimental plant was selected for BVW larvae infestation. Third-instar larvae were provided by Dr. Richard Cowles (Connecticut Agricultural Experiment Station, Windsor, Connecticut). On day 0, each experimental young ($N = 9$) and experimental mature ($N = 8$) plant received seven and six BVW larvae, respectively. Seven days after BVW larvae infestation, roots and shoots were harvested for 20E analysis. Soils were carefully sifted for root fragments and searched for the presence of live BVW larvae. Upon harvesting, two experimental plants (both in the mature group) completely lacked live BVW larvae, and these plants were removed from the final analysis. Low soil moisture was believed to be responsible for this unexpected mortality.

Phytohormone Effects on 20E Accumulation. Inducible plant secondary metabolites appear to be regulated by multiple hormonal factors, many of which are believed to act through the jasmonic acid (JA) pathway (Thornburg and Li, 1991; Peña-Cortés et al., 1991; Farmer and Ryan, 1992; Sano and Ohashi, 1995; O'Donnell et al., 1996). We examined the impact of multiple plant hormones and GRs on 20E levels to elucidate potential signals involved in the induction response. Seventy-five plants were divided among 15 groups ($N = 5$, 1.65 ± 0.04 g). The treatments and hydroponic solution concentrations created were as fol-

lows. A positive MJ control was tested at 0.5 μM, a level previously determined to cause a maximal level of induction (Schmelz et al., 1998). IAA and NAA were tested at 0.05 and 0.50 μM, while GA_3, ABA, and Z were tested at 0.05, 0.50, and 5.00 μM. All plants were harvested four days after initial treatments, and general observations of root morphology were recorded.

Biosynthesis of Root 20E in Excised Roots. In some plants, PE biosynthesis appears to be restricted to specific tissue types (Tomás et al., 1993; Grebenok and Adler, 1993). We first examined the 20E biosynthetic capacity of the roots due to the localization of the induction. Following from Grebenok and Adler (1993), 20E biosynthesis was investigated by using pulse-chase studies with the DBED salt of *RS*-[2-^{14}C]mevalonic acid ([^{14}C]MVA), which is one of the first dedicated precursors to terpenoid and steroid biosynthesis in plants. Six hydroponically grown plants were divided into two groups (N = 3, 5.21 ± 0.17 g). The plant root systems were gently placed into 20 ml scintillation vials carrying 2 ml of H_2O and 2 μCi of [^{14}C]MVA supplied by a 10-μl drop of methanol. In the first group, shoots were immediately excised from roots with a razor. In the second group, plants remained intact for 1 hr prior to shoot excision, with a greater root uptake of radiolabel anticipated due to shoot transpiration. The shoots harvested immediately at time 0 could not contain radiolabel, and thus were not analyzed. Shoots harvested at 1 hr were immediately frozen for analysis. All root samples were harvested 24 hr after the start of the experiment.

Biosynthesis of Root 20E in Intact Plants. Early experiments demonstrated that spinach roots are active sites of 20E biosynthesis. By using a [^{14}C]MVA pulse-chase design, we investigated induced root 20E biosynthesis as a potential mechanism of 20E induction following damage and MJ treatments. Fifteen hydroponic plants were divided into three groups (N = 5, 2.36 ± 0.09 g) consisting of untreated controls, MJ, and root damage treatments. To minimize ^{14}C contamination of the surroundings, two days prior to the beginning of the experiment, plants were removed from the growth chamber and maintained in a ventilated fume hood. Under these conditions, the original light–dark cycle and intensity (PAR) was maintained, with changes only in the ambient humidity (20%) and temperature (20°C). For the [^{14}C]MVA pulse, the root systems of all plants were gently placed on the bottom of individual plastic soufflé cups (29.6 ml; Solo-P100, Urbana, Illinois) with a minimal volume (3 ml) of water. [^{14}C]MVA (1 μCi in 10 μl methanol) was added to the aqueous solution of each cup. For the next 24 hr, plant solution volumes were maintained at this low level in an effort to maximize radiolabel uptake. Plants were removed, and the root systems were rinsed with double distilled water to remove residual radiolabel. Plants were returned to their original 1-liter hydroponic solutions. One hour after this step, MJ and root damage treatments were performed. Each MJ-treated plant received 5 ml of an aqueous MJ solution resulting in 0.90 μM final hydroponic solution concentration. The root damage (RD) treatment follows from Schmelz

et al. (1998), and was created by firmly squeezing sections of the root between the thumb and index finger. This produced an area of damage approximately 2 cm long and was repeated eight times to distribute damage equally along the length of the root system. All plants were harvested for analysis three days after induction treatments.

Statistical Analysis. An analysis of variance (ANOVA) was performed on root and shoot PE and biomass measures for all experiments. Significant treatment effects were investigated if the main effects of the ANOVA were significant ($P < 0.05$). Tukey tests were used to correct for multiple comparisons in experiments where no a priori hypotheses existed. Data analysis was accomplished with the aid of the MGLH module of the SYSTAT statistical package (Evanston, Illinois).

RESULTS

Root Excision and 20E Induction. By simply removing a range of root tissue, we examined the plant responses to damage that occur in the absence of herbivore-derived variables. Both the positive control (MJ) and 70% root removal treatments resulted in inductions (ANOVA $F_{4,25} = 26.87$, $P < 0.001$) of root 20E concentrations reflecting 2.6- and 6.0-fold increases, respectively (Figure 1). This robust response is also seen on a 20E pool size basis. The total 20E pool in the remaining roots of the 70% root removal group (6.3 ± 0.8 μg) was higher than the entire intact control (3.2 ± 0.5 μg) root pool (ANOVA $F_{4,25} = 5.87$, $P = 0.002$, protected contrast $F_{1,25} = 4.53$, $P = 0.043$). Lower root mass removal levels (15–35%) failed to produce any significant changes in root 20E concentrations. The 70% root removal treatment also resulted in a 1.5-fold increase (ANOVA $F_{4,25} = 6.41$, $P = 0.001$) in shoot 20E concentration (Figure 1). All other treatments failed to produce changes in shoot 20E concentrations. As expected, root removal treatments resulted in decreases (ANOVA $F_{4,25} = 18.98$, $P < 0.001$) in the root dry biomass remaining at the end of the experiment. At this point, the 70% removal group had less root mass (25 ± 3 mg) than all other treatment groups. Similarly, 35% root mass removal resulted in a final root mass (54 ± 4 mg) that was lower than the control (77 ± 7 mg) and MJ (81 ± 6 mg) groups (all Tukey $P < 0.05$). No differences were detected in shoot dry mass values (ANOVA $F_{4,25} = 2.33$, $P = 0.084$).

Insect Feeding and 20E Induction. To contrast mechanical damage, we examined the root 20E induction response of plants fed upon by BVW larvae. In young plants, herbivory resulted in a 50% decrease (ANOVA $F_{1,16} = 19.99$, $P < 0.001$) in root dry mass compared to the uninfested controls (Figure 2B). Concordantly, a 4.7-fold increase (ANOVA $F_{1,16} = 23.04$, $P < 0.001$) in root 20E concentrations above the uninfested controls was observed (Figure 2D). In a

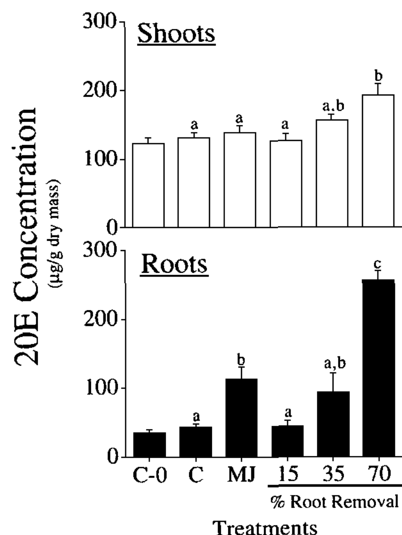

FIG. 1. Shoot and root 20E concentrations (+SEM) four days after excision of either 15, 35, or 70% of the root mass. Untreated control (C) and methyl jasmonate (MJ, 0.45 μM)-treated plants are included for induction comparisons. The day 0 control (C-0) was included to determine initial root mass removal levels but is not included in the statistical analysis. Within plots, bars that share the same letter are not significantly different ($P > 0.05$, Tukey correction for multiple comparisons).

more conservative measure of induction, the total root pool of 20E in the BVW larvae-infested group (5.42 ± 0.64 μg) was still larger (ANOVA $F_{1,16} = 9.35$, $P = 0.008$) than the uninfested control group (2.55 ± 0.67 μg). Thus, even though herbivory reduced the root mass by 50%, the total pool of 20E present in the remaining root tissue was over 2.0-fold higher than that found in the intact control roots. Shoot dry mass and shoot 20E concentrations did not differ in the young plants (both ANOVAs $F_{1,16} < 4.02$, $P > 0.062$: Figure 2A and C).

In mature plants, no changes in root or shoot dry mass were detected (both ANOVAs $F_{1,12} < 0.92$, $P > 0.350$; Figure 2A and B). Even though herbivory did not result in statistically detectable losses in root mass, plants fed upon by BVW larvae demonstrated 3.0-fold increases (ANOVA $F_{1,12} = 19.77$, $P = 0.001$) in root 20E concentrations above those of the uninfested controls (Figure 2D). The average root 20E pool sizes of the control and BVW larvae infested groups were 10.71 ± 2.77 μg and 28.72 ± 9.32 μg, respectively. This represents an average 2.7-fold increase in the total root 20E pool; however, due to the variability in total root mass observed among mature plants, this measure was not significant (ANOVA $F_{1,12} = 4.40$, $P = 0.058$). As with the young plants, no differences

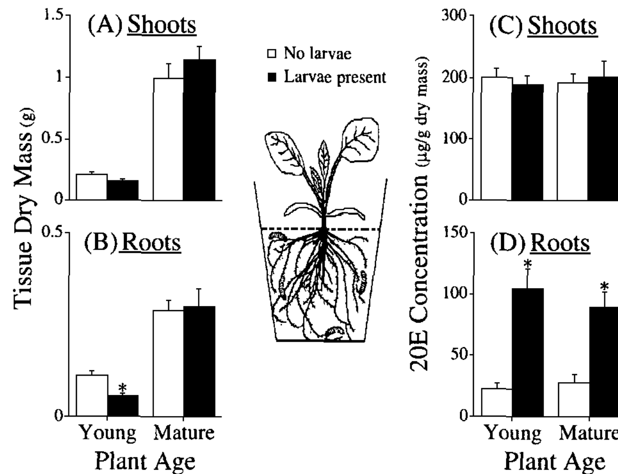

FIG. 2. Mean (+SEM) (A) shoot and (B) root tissue dry mass (g) and (C) shoot and (D) root 20E concentrations, after seven days of infestation by black vine weevil (BVW, *Otiorhynchus sulcatus*) larvae. Black and white bars represent the presence and absence of BVW larvae, respectively. Asterisks denote significant differences from the paired controls (ANOVA $P < 0.05$).

in shoot 20E concentrations were detected (ANOVA $F_{1,12} = 0.62$, $P = 0.445$: Figure 2C).

Phytohormone Effects on 20E Accumulation. We compared the 20E inducing activity of NAA, IAA, GA_3, ABA, and Z to that of MJ. Increases (ANOVA $F_{14,60} = 8.97$, $P < 0.001$) in root 20E concentrations were induced by both MJ (0.5 μM) and NAA (0.5 μM), resulting in 4.8- and 5.7-fold increases, respectively (Figure 3C). High levels of ABA (5.0 μM) appeared to have some 20E-inducing activity; however, the resulting root 20E concentrations were intermediate and not significantly different from either the control or MJ treatments. Treatments involving IAA, GA_3, and Z had no effect on root 20E levels at the concentrations tested. Increases (ANOVA $F_{14,60} = 9.36$, $P < 0.001$) in shoot 20E concentrations were induced by treatments with ABA (5.0 μM) and Z (0.5 and 5.0 μM) resulting in 1.7-, 1.5-, and 1.6-fold increases over untreated controls, respectively (Figure 3B). No other treatments caused alterations in shoot 20E concentrations. Total root and shoot dry biomass values were unaltered by the treatments (both ANOVAs $F_{14,60} < 0.92$, $P > 0.545$). However, increases (ANOVA $F_{14,60} = 16.90$, $P < 0.001$) in the root–shoot dry mass ratios indicated that a change in relative growth and resource allocation had occurred. The NAA (0.05 and 0.50 μM) and Z (5.0 μM) treatments resulted in higher root–shoot dry mass ratios than those of the untreated control group, corresponding to 1.3-,

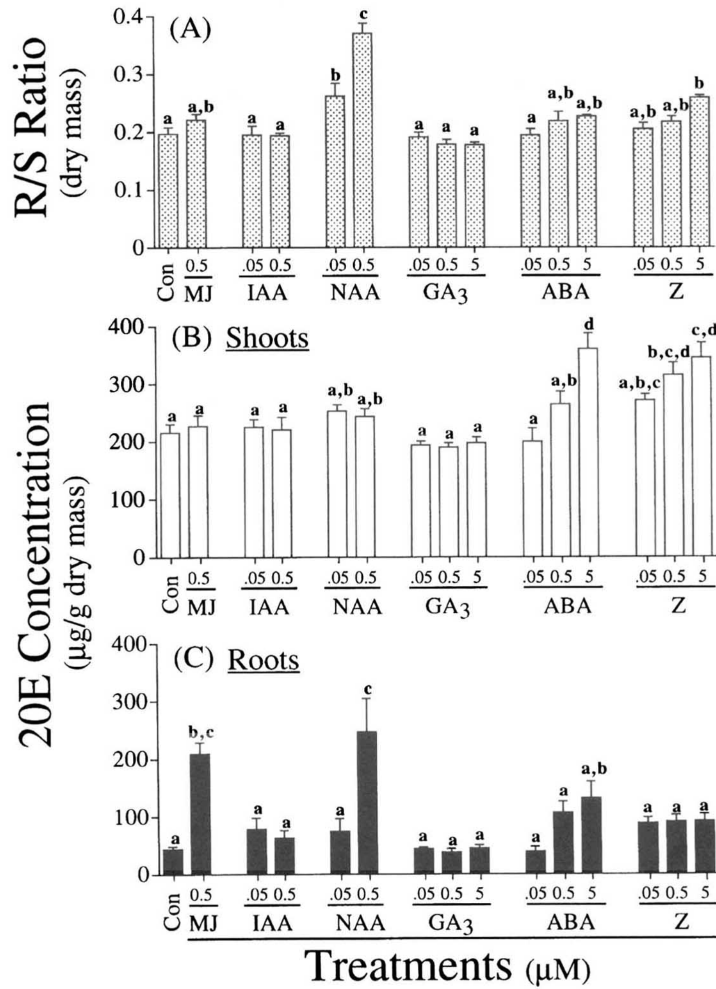

FIG. 3. Mean (+SEM) (A) Root–shoot dry mass ratios, (B) shoot and (C) root 20E concentrations of spinach plants following four days of hydroponic growth in solutions containing either methyl jasmonate (MJ), indole-3-acetic acid (IAA), 1-naphthaleneacetic acid (NAA), gibberellic acid (GA_3), abscisic acid (ABA), or *trans*-zeatin (Z). Labels on the *x*-axis denote the initial micromolar concentrations of phytohormones and growth regulators used. All control plants (Con) were untreated. Within plots, bars that share the same letter are not significantly different ($P > 0.05$, Tukey correction for multiple comparisons).

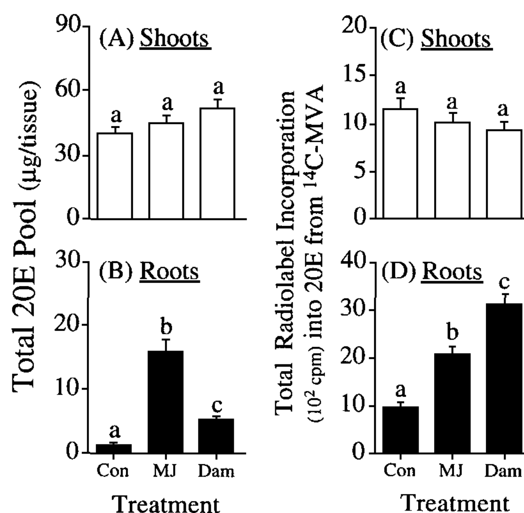

FIG. 4. Mean (+SEM) (A) shoot and (B) root 20E pools (μg/tissue) three days after root damage (Dam) and methyl jasmonate (MJ) treatments. Total shoot (C) and root (D) [^{14}C]MVA incorporation into the corresponding 20E pools following Dam and MJ treatments is shown. Control plants (Con) were subjected only to [^{14}C]MVA additions. Within plots, bars that share the same letter are not significantly different ($P > 0.05$, Tukey correction for multiple comparisons).

1.9-, and 1.3-fold increases, respectively (Figure 3A). Observations of root morphology suggested that the NAA (0.5 μM) treatments produced large increases in lateral root formation and root cross-section diameters. Small increases in the number of lateral roots were also evident in the NAA (0.05 μM) and Z (0.05–0.5 μM) treatments. All other treatments produced roots that were within the variation observed for untreated plants.

Root 20E Biosynthesis and Mechanism of Induction. We investigated the potential of root 20E biosynthesis in an effort to understand the origin of root 20E accumulation. A 24-hr incorporation of [^{14}C]MVA into excised spinach roots resulted in 1623 ± 379 cpm/μg 20E for immediately excised roots and 6518 ± 1293 cpm/μg 20E for the 1-hr excision group. Corresponding shoots from the 1-hr excision group incorporated only 4 ± 1 cpm/μg 20E. Active root synthesis of 20E appears to be the most parsimonious explanation for these patterns.

We examined the potential of induced root 20E synthesis as a possible mechanism for induced root 20E accumulation. Both MJ and damage caused increases in root 20E pools and ^{14}C incorporation into those pools (both ANOVAs $F_{2,12} > 27.21$, $P < 0.001$; Figure 4B and D). MJ and damage resulted in 11.1- and 3.8-fold increases in total root 20E pools, respectively. The cor-

responding MJ- and damage-induced increases in ^{14}C radiolabel incorporated into these same 20E pools were 2.2- and 3.3-fold, respectively. Overall, a trend is apparent for increases in root de novo synthesis of 20E; however, shoot 20E pools and ^{14}C incorporation into shoot 20E did not differ (both ANOVAs $F_{2,12} < 2.86$, $P > 0.095$: Figure 4A and C). Likewise, root damage and MJ treatments had no effect on final root or shoot dry biomass (both ANOVAs $F_{2,12} < 1.72$, $P > 0.220$).

DISCUSSION

Our results lead to the following conclusions. First, high levels of root removal (70%) result in significant root 20E induction responses, while lower removal levels (15–35%) have no effect. Shoot 20E induction is detected only under the most severe conditions of root damage. Second, regardless of the amount of root tissue lost, root herbivory by BVW larvae resulted in a 3.0-fold induction of root 20E concentrations. Thus, under natural conditions, even low levels of root tissue removal by insect herbivory are sufficient to signal this response. Third, MJ is the most active of the natural phytohormones tested in stimulating root 20E accumulation. This strengthens support for a role of endogenous jasmonates in signaling this response. Fourth, spinach roots are active sites of 20E biosynthesis. Damage and MJ treatments caused increases in [^{14}C]MVA incorporation into the induced 20E pools, indicating that increased de novo root biosynthesis of 20E is an important mechanism behind the induction phenomenon.

Investigations of plant physiological responses to damage often utilize tissue excision as a simple and reproducible treatment to approximate insect herbivory (Welter, 1991). While such treatments have obvious methodological advantages, they also lack important complexities existing in plant–insect interactions (Baldwin, 1990). The temporal differences in wounding between root excision and insect feeding are extreme. Root excision results in a single damage event that likely corresponds to a single burst of wound signal production (Creelman et al., 1992; Baldwin et al., 1997). In contrast, herbivory by numerous BVW larvae is likely to be a continuous process and one that results in a sustained increase in wound signal production (for an example involving *Manduca sexta*, see McCloud and Baldwin, 1997). The response of plants to insect oral secretions represents another difference between mechanical damage and actual herbivory. In corn, the release of parasitoid-attracting volatiles, following beet armyworm feeding, cannot be mimicked by mechanical damage alone (Turlings et al., 1993). Volicitin (N-17-hydroxylinolenoyl-L-glutamine), present in beet armyworm oral secretions, elicits the release of a specific blend of volatiles by the plant (Alborn et al., 1997). We found that when mature soil-grown plants were infested by BVW larvae, a root 20E induction response was stimulated,

even though no significant root loss was detected. Conversely, in hydroponically grown spinach plants, root mass excision of greater than 35% was required to elicit a root 20E induction response. This disparity probably reflects differences in temporal or spatial damage patterns that influence endogenous wound signal production (Ohnmeiss et al., 1997). While insect-derived elicitors may influence this response, they are not necessary, as mechanical damage alone can induce root 20E accumulation.

Based on the activities of the natural phytohormones tested, jasmonic acid remains the best candidate signal for mediating the induction of root 20E concentrations following real and simulated root herbivory. While the activity of the synthetic auxin NAA is comparable to MJ, numerous studies suggest that the actions of exogenous NAA may be artifactual from an ecological perspective. Blakely et al. (1988) found NAA to have a 30-fold higher activity than IAA in the induction of lateral root formation. In tomato, NAA causes the induction of lateral root formation that is correlated with the accumulation of proteinase inhibitor (Pin) II family transcripts (Taylor et al., 1993). This response appears to be uncharacteristic following root mechanical damage and nematode attack, as both failed to induce root Pin II accumulation but did result in systemic foliar Pin II increases (Peña-Cortés et al., 1988; Hammond-Kosack et al., 1990). We hypothesize that the NAA-induced (0.5 μM) accumulation of root 20E is an indirect result of increased lateral root formation. Our morphological observation is supported by a significant 1.9-fold increase in the root–shoot ratio. Because PEs normally occur at higher concentrations in growing root tips (Dinan, 1995), it seems possible that the NAA-induced root 20E concentrations are simply a result of this change in root morphology. However, there is one caveat to this argument: treatments that produced only small increases in both lateral root formation and root–shoot ratios did not result in significant root 20E inductions.

Severe root damage, but not MJ treatments, stimulated significant increases in shoot 20E concentrations. This suggests that shoot 20E dynamics are controlled by additional signals or indirect physiological effects following root stress. Two candidate phytohormones that are able to alter foliar 20E concentrations are abscisic acid and the cytokinin, *trans*-zeatin. Endogenous ABA levels are often induced after damage (Peña-Cortés et al., 1989) and are correlated with activating numerous stress-responsive genes. However, the situation is complex, because stress-induced increases in ABA are not always correlated with wound gene activation (Peña-Cortés and Willmitzer, 1995). For damage-induced responses, ABA is believed to act upstream in the signal cascade, leading to jasmonic acid synthesis (Peña-Cortés et al., 1995, 1996). More recently, however, evidence suggests that a JA-independent pathway exists for ABA signaling following wounding (Dammann et al., 1997). Elevations in shoot 20E concentrations following root damage appear to be the result of a general stress response, possibly involving ABA, but not directly caused by jasmonate signals. Moderate

foliar increases in 20E were also stimulated by Z at the two highest concentrations tested. Cytokinins have been implicated in mediating plant responses and signal transduction pathways following damage and pathogen attack (Sano and Ohashi, 1995). Unlike ABA and Z, GA_3 treatments did not impact root or shoot 20E accumulation patterns. An understanding of the roles that cytokinins and gibberellins play in modulating defensive reactions in plants is still in its infancy (Sano and Ohashi, 1995; Jacobsen and Olszewski, 1996).

Secondary metabolite production in plants is often limited to specific tissue and cell types, with transport processes allowing alternative sites of accumulation. This general pattern exists in some PE-producing plants; for example, PE biosynthesis in *Ajuga reptans* occurs only in the roots (Tomás et al., 1993). Our results indicate that excised roots incorporate radiolabel from [^{14}C]MVA into 20E within 24 hr. This supports the idea that PE biosynthesis occurs in both roots and shoots of spinach. In our concluding experiment, the mirror increase of the damage-induced root 20E pool (3.8-fold) with that of the ^{14}C-radiolabeled 20E (3.3-fold), supports de novo root biosynthesis as the source of wound-induced 20E accumulation. A root 20E induction mechanism involving shoot 20E synthesis and subsequent basipetal transport would predict an increase in shoot ^{14}C-radiolabeled 20E. Total ^{14}C incorporation into shoot 20E pools was unaffected by damage and MJ treatments. Thus, wound-induced increases in root 20E levels appear to be caused by increases in root biosynthesis.

Jasmonic acid is known to signal many plant defense responses following mechanical damage (Karban and Baldwin, 1997). However, when MJ is used experimentally, in isolation from other signaling complexities associated with damage, wound responses may not be effectively reproduced (Singh et al., 1998). Unlike mechanical damage, we found that only 20% of the MJ induced root 20E pools can be explained by increases in de novo biosynthesis as measured by [^{14}C]MVA incorporation. We offer two alternative explanations for this discrepancy. In plants, terpenoids are derived from both mevalonate-dependent and mevalonate-independent pathways (Arigoni et al., 1997). The [^{14}C]MVA pulse only estimates 20E biosynthesis through the mevalonate-dependent pathway. Examination of the mevalonate-independent pathway requires the use of ^{14}C-labeled 1-deoxy-D-xylulose as a pathway precursor. MJ may preferentially stimulate the mevalonate-independent pathway; however, the effect of jasmonates on this pathway are unknown. A second alternative is the potential conversion of previously synthesized membrane sterols, for example, spinasterol and 22-dihydrospinasterol, into 20E. This possibility is considered because root spinasterol and 22-dihydrospinasterol pools are often reduced in plants that have undergone a large root 20E induction (Schmelz and Grebenok, unpublished data). The release of free 20E from conjugates is an unlikely mechanism for driving induced 20E accumulation, since levels of 20E conjugates reported in spinach are extremely low (Grebenok et al., 1994).

Karban and Baldwin (1997) summarize our collective knowledge of induced plant-resistance to root feeding insects by stating that the phenomenon "has been pretty much undescribed." Part of this problem involves developing an understanding of the chemical changes that occur in roots following herbivory. Our current work adds to the few related studies in this area that include furanocoumarin induction in parsnip roots (Zangerl and Rutledge, 1996), induced chitinases in citrus roots following herbivory by weevil larvae (Mayer et al., 1995), and the induction of root glucosinolates in *Brassica napus* following herbivory by turnip root fly maggots (Birch et al., 1990). We demonstrate that the wound- and insect-induced accumulation of root 20E is an active process that requires increases in de novo biosynthesis. The wound signal analog MJ can be used as a tool for the manipulation of root 20E levels in growing plants; however, some differences between MJ- and damage-induced responses appear to exist. The ecological significance of induced 20E levels is currently being investigated. In future experiments, root damage and MJ treatments will be used to generate root tissues of varying 20E concentrations for tests involving root herbivore preference and performance. These experiments may finally begin to test whether endogenous PEs actually function as plant defense compounds in natural systems (Galbraith and Horn, 1966).

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CROSS-ATTRACTION OF *Carpophilus humeralis* TO PHEROMONE COMPONENTS OF OTHER *Carpophilus* SPECIES

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Abstract—The pineapple beetle, *Carpophilus humeralis*, is known from field tests to be more attracted to baits containing the pheromone blends of other *Carpophilus* species and food odors than to food odors alone, but a pheromone is not yet known for *C. humeralis*. Wind-tunnel bioassays were used to determine specifically which of the *Carpophilus* pheromone components were the most attractive for *C. humeralis*. Nine tetraene and triene components normally used in field studies, plus eight additional male-specific compounds from other *Carpophilus* species, and one additional analog, were used in these experiments. At the 10-ng level, 15 of 18 compounds tested with a food-related coattractant (propyl acetate) were more attractive for *C. humeralis* than the coattractant alone. With decreasing doses (1, 0.1, and 0.01 ng) the number of attractive compounds declined. Only one compound, (2*E*,4*E*,6*E*,8*E*)-3,5,7-trimethyl-2,4,6,8-decatetraene (**1**), had significant attraction for *C. humeralis* at 0.01 ng, close to the lower threshold level of pheromone attraction for other *Carpophilus* species. Responses to mixtures of compounds were explored. For example, **1** was synergistic with 4-ethyl-2-methoxyphenol (E), 2,5-diisopropylpyrazine (D), and 2-phenylethanol (P), which are known attractants for *C. humeralis;* a mixture of **1** plus E, D, and P was 20 times more attractive than **1** alone and five times more than E, D, plus P. The specificity of *C. humeralis* for certain tetraenes was similar to that exhibited by *C. hemipterus*, while the specificity for trienes was similar to that of *C. freemani*. The behavior of *C. humeralis* to these pheromone components suggests that it may have a pheromone like that of other *Carpophilus* species or that it could possibly use these compounds as host finding kairomones.

Key Words—*Carpophilus humeralis*, pheromone components, structure-ac-

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tivity relationships, field trials, kairomones, wind tunnel bioassay, synergism, 4-ethyl-2-methoxyphenol, 2,5-diisopropylpyrazine, 2-phenylethanol.

INTRODUCTION

Sap beetles are pests in a wide variety of agricultural products, causing economic damage either directly by feeding or indirectly through the vectoring of rot-causing microorganisms (Hinton, 1945). One member of this complex of insects is the pineapple beetle, *Carpophilus humeralis* (F.) (Coleoptera: Nitidulidae), a serious pest of numerous fruits and vegetables (Gillogly, 1962). The need to control *Carpophilus* species, including the pineapple beetle, with less reliance on insecticides has led to research on attractants for these insects.

Recently, aggregation pheromones have been discovered for a number of nitidulid species, including: *C. hemipterus* (L.), *C. freemani* Dobson, *C. lugubris* Murray, *C. mutilatus* Erichson, *C. antiquus* Melsheimer, *C. obsoletus* Erichson, *C. davidsoni* Dobson, *C. dimidiatus* (F.), and *C. brachypterus* Say. Chemically, *Carpophilus* pheromones are blends of unsaturated hydrocarbons with alkyl branching and three or four conjugated double bonds. Field tests have demonstrated the ability of these pheromones to attract *Carpophilus* species in large numbers, especially when the pheromones are combined with fermenting food materials. *Carpophilus* species often share some pheromone components, and cross-attraction among species is common, although each species typically responds best to its own unique blend (Bartelt, 1997).

Attempts to identify a pheromone for *C. humeralis* have been unsuccessful. There was no chromatographic or behavioral evidence of sex-specific pheromone emission when *C. humeralis* was held on artificial diet, a procedure that was successful for other *Carpophilus* species (Bartelt et al., 1993b), or when natural host materials were used (Zilkowski et al., 1999; and unpublished data). Nevertheless, *C. humeralis* was significantly attracted to the synthetic pheromone blends for *C. hemipterus*, *C. obsoletus*, *C. lugubris*, *C. dimidiatus*, *C. freemani*, and *C. mutilatus* in field trials (Bartelt et al., 1992a, 1994, 1995a,b; Blumberg et al., 1993; James et al., 1993, 1994). All of these pheromone formulations were various subsets of nine key components specific to male *Carpophilus* beetles. Furthermore, the attraction of *C. humeralis* to these compounds is enhanced when combined with host-related attractants, such as decaying grapefruit (Blumberg et al., 1993) or fermenting bread dough (Bartelt et al., 1995b). In addition, Zilkowski et al. (1999) found that a mixture of 4-ethyl-2-methoxyphenol, 2,5-diisopropylpyrazine, and 2-phenylethanol, produced by microbes on fruit, acts synergistically with other fermenting food odors and the pheromones of *C. hemipterus* or *C. freemani* to enhance the attraction of *C. humeralis* in the field.

We wished to know specifically which *Carpophilus* pheromone compo-

nents were the most attractive for *C. humeralis*. All nine of the *Carpophilus* pheromone components used in previous field tests, eight additional male-specific compounds from these species, and one additional analog, not identified from any species, were investigated as attractants for *C. humeralis*.

METHODS AND MATERIALS

Beetle Cultures. The culture of *C. humeralis* originated from a date garden near Oasis, California. Beetle rearing was as described by Dowd and Weber (1991), using a modified diet (Bartelt et al., 1993b). Adults from the culture were used for wind tunnel bioassays.

Synthetic Compounds. Carpophilus pheromone components, compounds **1–17** (Table 1), had been prepared in previous work (Bartelt et al., 1990a-c, 1991, 1992b). Compound **18**, which is not known to exist in any *Carpophilus* species, was included to further explore structure-activity relationships involving triene compounds (**13–17**). Purity of compounds **1**, **3–7**, and **13–18** was >95%. The other compounds (**2**, **8-12**), which had been in storage longer, had purities ranging from 70% to 85%. The greatest percentage of these impurities consisted of *Z* isomers, and none of the impurities ever included any of the other numbered compounds. Synthetic all-*E* tetraenes isomerize slowly in storage, and in previous studies these *Z* isomers were not active for *C. hemipterus* in bioassays (Bartelt et al., 1992b). 4-Ethyl-2-methoxyphenol (E) (98%) was purchased from Lancaster Synthesis Inc., Windham, New Hampshire. 2-Phenylethanol (P) (99%) and propyl acetate (98%) were obtained from Aldrich Chemical Co., Milwaukee, Wisconsin. 2,5-Diisopropylpyrazine (D) (90%) had been prepared in previous work (Zilkowski et al., 1999). All chemicals were stored at −5°C when not in use.

Wind-Tunnel Bioassays. The attractiveness of test compounds for *C. humeralis* was assessed by wind-tunnel bioassay (Zilkowski et al., 1999). Briefly, approximately 500–2000 *C. humeralis* adults (sexes not determined), 1–2 weeks old, were placed into the wind tunnel. In 2–3 hr, beetles would begin to fly, and bioassays would start when 100 or more beetles were consistently in flight. Insects would remain responsive for periods of up to 4 hr. Treatments (in hexane) or hexane solvent alone (as the control), were applied (usually 10 μl) to 7-cm filter paper disks folded into quarters and suspended at the upwind end of the tunnel. These baits were always tested in pairs, hung side by side and separated from one another by 0.3 m. The total number of insects that landed on each treatment and control during a 3 min period was recorded. Each 3-min period was considered to be one test. There was a 2- to 4-min pause between tests while the next paper disks were prepared. Treatment locations were alternated for subsequent replications to avoid wind-tunnel position effects. Typically, 10–20

tests were conducted each day, although as many as 35 tests were performed on some bioassay days.

Unless otherwise stated, propyl acetate was added to each treatment and control (~20 μl of 10% propyl acetate in mineral oil, applied to one edge of the filter paper circle away from the treatment solution). As with other *Carpophilus* species (Dowd and Bartelt, 1991), *C. humeralis* responds to blends of small esters and fermentation volatiles; one of these, propyl acetate, has been routinely used as a bioassay coattractant because it strongly magnifies the effects of many other compounds but is only slightly attractive by itself (Zilkowski et al., 1999).

Comparisons among three or more treatments, which included all possible two-way tests, were done by using a balanced incomplete block design. Each 3-min test was considered to be a block. For statistical analysis, $\log(X + 1)$ transformation of the data was employed to stabilize variance. Analysis of balanced incomplete block designs was by the method of Yates (1940). Statistical differences among log-transformed means were determined by the least significant difference (LSD) method.

There were too many pheromone components and doses to be evaluated in one bioassay day. Therefore, each pheromone component (plus propyl acetate) was compared to the control (plus propyl acetate) and to an attractive standard in a small, balanced, incomplete block design. With this design, the attractive standard was also compared to the control. Each pair of treatments was tested four times; thus, there were eight replications for each treatment. The attractive standard, chosen based on initial screenings (unpublished results), was 10 ng of compound **13** (plus propyl acetate). The relative activity of each pheromone component was calculated as an index of activity (IA), which expressed the attraction of each component as a percentage of that for compound **13** (corrected for the control response) according to the following equation:

$$\mathrm{IA} = \frac{(\text{Pheromone component} - \text{Control})}{(\text{Compound 13} - \text{Control})} \times 100$$

An IA equal to 0 indicates activity at the control level, and 100 indicates activity as for compound **13**. The IA of each pheromone component was first determined by using 10 ng/test, a level commonly used for the other *Carpophilus* species pheromone bioassays (Bartelt, 1997). Compounds that had IA > 10 and were significantly different from the control at the $P = 0.001$ level were diluted 10-fold and tested again. This was followed by further dilution and retesting until the difference was not significant at $P = 0.001$ or the IA was <10.

Test for Synergistic Effects Among Carpophilus *Male Produced Compounds.* Synergistic attraction for *C. freemani* has been demonstrated to a combination of the most abundant triene and tetraene components found in the pheromone blend

of this species (Bartelt et al., 1990b). The possibility of synergistic activity from a combination of the most active tetraene and triene components was explored for *C. humeralis* with wind-tunnel bioassays. Dose levels of 1 and 0.1 ng of compounds **1** and **13** were tested.

Synergism of Compound 1 with Host-Related Volatiles. The pheromones of many *Carpophilus* species are synergized by fermenting food materials (Bartelt, 1997). Wind-tunnel bioassays were performed to determine if host-related volatiles that are particularly attractive to *C. humeralis*, 4-ethyl-2-methoxyphenol (E) (20 ng), 2,5-diisopropylpyrazine (D) (1 ng), and 2-phenylethanol (P) (35 ng) (Zilkowski et al., 1999) could synergize the attraction of the most active pheromone component. Pairwise comparisons were made among: compound **1**; a mixture of E, D, and P; a combination of compound **1** plus the mixture of E, D, and P; and an appropriate control. In addition, a series of bioassays was done to determine which compounds or combinations of E, D, and P gave the greatest attraction when combined with the most active pheromone component. Experiments were performed comparing all combinations of E, D, and P plus compound **1**, against a mixture of all four compounds. Each treatment was tested 6 times, both with and without the propyl acetate coattractant. The balanced incomplete block design was used for these experiments, and the results were expressed as an index of activity (IA). For these experiments, the IA was defined as:

$$\mathrm{IA} = \frac{\text{Test blend} - \text{Control}}{(\mathrm{E} + \mathrm{D} + \mathrm{P} + 1) - \text{Control}} \times 100$$

By standardizing the effect of each combination as an IA, it was easier to evaluate the impact each compound had in the attraction of the total blend. Otherwise, day-to-day variability in absolute beetle responsiveness would have obscured trends among treatments.

RESULTS

Activity of Pheromone Components Tested Alone. The IAs of compounds tested at levels of 10, 1, 0.1, and 0.01 ng are shown in Table 1. The overall mean for the propyl acetate control was 1.5 landings per test (37 experiments, eight replications per experiment), and the means for the 37 individual experiments ranged from 0.3 to 4.5. For the attractive standard (10 ng of **13** plus propyl acetate), the overall mean was 29.0, and the means for single experiments ranged from 10.7 to 116.4. All but one of the 12 tetraene compounds tested (**12**) was more attractive than the propyl acetate control, and compounds **1–7** had greater attraction than compound **13**. Similarly, four of the six triene compounds (**13–16**), tested at 10 ng, were more active than the control, but none of the other trienes (**14–18**) approached the attractiveness of **13**.

TABLE 1. COMPOUND STRUCTURES, *C. humeralis* INDICES OF ACTIVITY (SEE METHODS AND MATERIALS), AND *Carpophilus* SPECIES FROM WHICH EACH COMPOUND HAS BEEN IDENTIFIED

Compound	Structure	Index of activity per dose (ng)[a]				*Carpophilus* species[b]
		10	1	0.1	0.01	
1		361.3***	199.5***	45.0***	2.8*	**H**,[c] **B**,[c] D
2		365.9***	81.6***	25.0*		**H**, **B**, D
3		370.1***	61.4***	13.0**		**H**, **L**,[c] **F**, **B**, **D**
4		302.0***	77.6***	14.5*		**H**, **L**, F, **B**, **D**, **O**[c]
5		154.0***	38.4***	4.7		D
6		140.0***	19.1*			D, **A**,[c] **Di**[c]
7		201.3***	11.3**			F, D
8		58.0***	4.6*			H, **B**
9		34.4***	2.6			H, D
10		22.5***	3.7			H
11		16.7***	9.5*			H
12		1.9				H
13[d]		100.0***	8.3***			H, **F**,[c] **D**[c]
14		13.5***	2.6			F, **D**, **M**
15		4.6*				F, D, **M**[c]
16		2.1*				D, F
17		2.0				D, F
18		3.8				None

[a]*, **, and *** denote significant differences from the propyl acetate control at the 0.05, 0.01, and 0.001 levels respectively [*t* tests in the log (X + 1) scale].

[b]*Carp.* species: H = *hemipterus*, L = *lugubris*, F = *freemani*, B = *brachypterus*, D = *davidsoni*, O = *obsoletus*, M = *mutilatus*, A = *antiquus*, Di = *dimidiatus*.

[c]Most abundant component in species pheromone (Bold letters indicate component normally included in synthetic pheromone for species).

[d]Compound **13** (10 ng) used as the standard high control (see Methods and Materials).

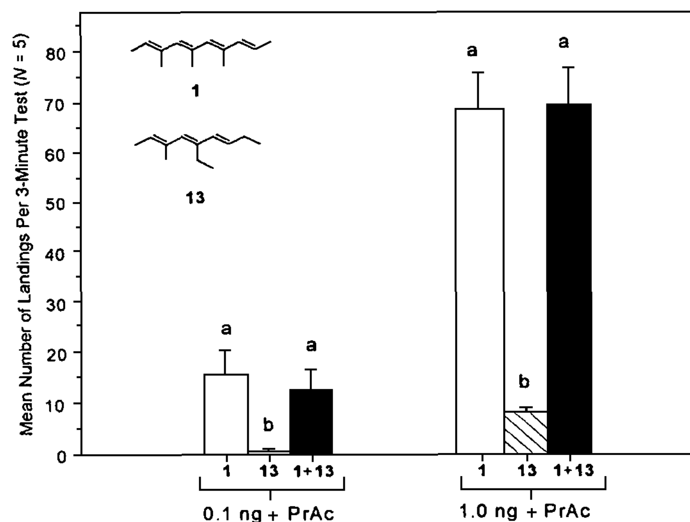

FIG. 1. Wind-tunnel bioassay results for the test of synergism of tetraene compound **1** with triene compound **13** at 0.1 ng and 1 ng levels. Within each group, treatments without letters in common differ significantly [LSD, 0.05, ANOVA in log $(X + 1)$ scale].

Although **1** was matched in activity at the 10 ng level by **2**, **3**, and almost by **4**, it was the most attractive compound when tested at lower doses. At the 1-ng level, **1** had an IA at least twice that of any other compound and was the only one more active than the standard. At the 0.1 ng level, **1** had an IA about half the standard but was almost twice as attractive as any other compound tested at the same level. At the 0.01 ng level, **1** was still more attractive than the propyl acetate control ($t = 3.01$, $P = 0.013$).

Tests for Synergism. Bioassays of the most attractive tetraene (**1**) combined with the most attractive triene (**13**) showed no signs of synergism (Figure 1). At both the 1-ng and 0.1-ng level, **1** was more attractive than **13**, but no difference in attraction was observed between **1** and a combination of **1** and **13** at either dose level.

Synergistic effects were seen when compound **1** was combined with the E, D, and P mixture (Figure 2, first group of bars in both panels, summarized as indices of activity relative to the whole mixture of **1** plus E, D, and P, both with and without propyl acetate). Without propyl acetate, **1** plus E, D, and P had a fivefold greater attraction in wind tunnel bioassays than E, D, and P alone and a 20-fold increase in attraction over compound **1** alone. With propyl acetate, **1** plus E, D, and P attracted three times more beetles than E, D, and P alone and five times more than compound **1** alone.

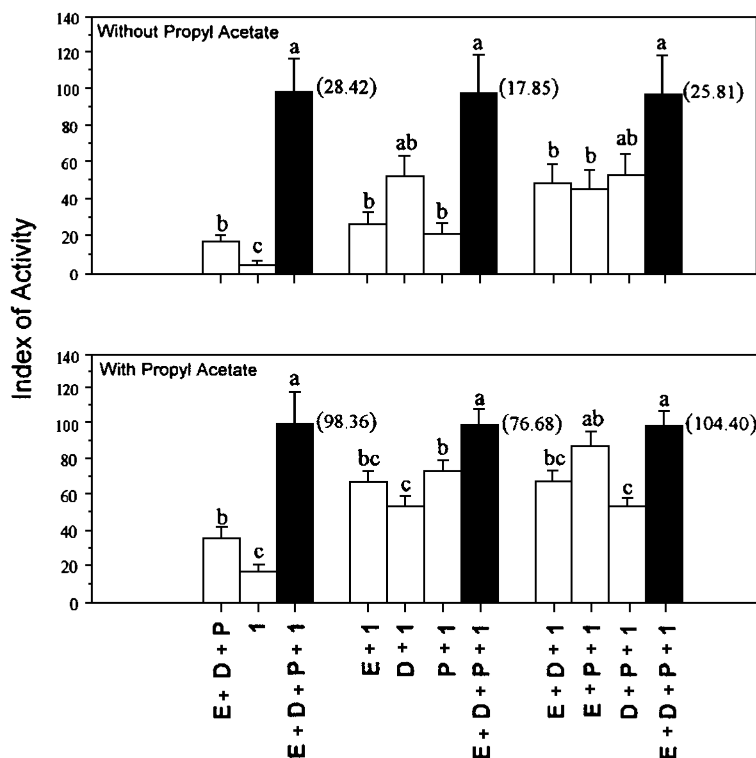

FIG. 2. Wind-tunnel bioassay results for all combinations of E, D, and P plus compound **1**, without and with propyl acetate coattractant. Bioassay results are expressed as an index of activity (see text). E = 4-ethyl-2-methoxyphenol (20 ng), D = 2,5-diisopropylpyrazine (1 ng), and P = 2-phenylethanol (35 ng). Within each group of three or four bars, treatments without letters in common differ significantly [LSD, 0.05, ANOVA in log $(X + 1)$ scale]. Black bars represent the treatment that defines IA = 100. Numbers in parentheses are the mean number (N = 6) of beetle landings per 3-min test period.

Individual and pairwise effects of E, D, and P on the activity of compound **1** are presented in Figure 2 as indices of activity. With or without propyl acetate, the IA for **1** plus E, D, or P was higher than the IA for **1** by itself. In most cases, the addition of any single compound to **1** was not enough to match the attractiveness of the complete blend. However, the combination of D + **1** without propyl acetate was not significantly different from the complete blend (t = 2.20, P = 0.055), although its IA was only just over 50%.

Without the propyl acetate coattractant, adding any two components from the attractive mix resulted in similar IAs among those treatments. Although D

+ P + **1** had an IA 45% less than the complete blend, the difference was not significant (t = 2.11, P = 0.064).

With propyl acetate, none of the pairs of compounds from the E, D, and P mixture added to **1** had an IA equal to that of the complete blend, although the treatment containing E + P + **1** with propyl acetate was not different from it (t = 1.01, P = 0.339). After **1** was combined with a single compound from the E, D, and P mixture, adding a second component typically resulted in only a slight change in the IA.

DISCUSSION

The response of *C. humeralis* to heterospecific pheromone components in wind-tunnel bioassays is consistent with the cross-attraction of this beetle in field experiments to the four-component *C. hemipterus* pheromone blend (**1**, **2**, **3**, and **4**) (Bartelt et al., 1992a, 1994, 1995a,b; Blumberg et al., 1993; James et al., 1993, 1994), the two-component *C. lugubris* pheromone blend (**3** and **4**) (Blumberg et al., 1993; Bartelt et al., 1994, 1995b), and the single-component *C. obsoletus* pheromone blend (**4**) (Bartelt et al., 1994, 1995a,b). The pheromone blend of *C. dimidiatus* (**6**, with a small amount of **14** included as an artifact of synthesis) also attracted *C. humeralis* in the field (Bartelt et al., 1995b), consistent with the moderate potency of **6** in the wind-tunnel bioassays. The attraction of *C. humeralis* in the field to the two-component pheromone blend of *C. freemani* (predominantly **13**, with a small amount of **3**) was generally less than to the other pheromone blends above (Blumberg et al., 1993; Bartelt et al., 1994, 1995a,b). Similarly, **13** was only moderately attractive to *C. humeralis* in wind-tunnel bioassays. Low catches of *C. humeralis* in the field to the two-component pheromone blend of *C. mutilatus* (**14** and **15**) (Bartelt et al., 1994, 1995a,b; Blumberg et al., 1993; James et al., 1993, 1994) are in agreement with the low potency of **14** and **15** in the wind-tunnel bioassays.

Based on wind-tunnel responses, some generalizations can be made about the structure–activity relationships for *C. humeralis* attraction: (1) Tetraenes are usually more attractive than trienes. (2) The right-hand terminal alkyl group of the tetraenes (Table 1) can be either methyl or ethyl and still have good activity (e.g., **1**, **2**, **3**, **4**). (3) The right-hand alkyl branch of the tetraenes can be either methyl or ethyl and still have strong attraction (e.g., **1**, **2**, **3**, **4**). (4) Tetraenes that have ethyl as the left-hand terminal alkyl group are less attractive than those with a methyl group (e.g., **8** vs. **1**, **9** vs. **4**). (5) Tetraenes with an ethyl group as the middle alkyl branch are almost always less attractive than the corresponding ones with a methyl group (e.g., **10** vs. **2**, **12** vs. **1**). (6) Any alterations to the basic triene structure of compound **13** decreases its attractiveness, such as adding carbons (**14**, **15**, **18**) or removing them (**16**, **17**). (Compound **18** has not been

detected from any *Carpophilus* species, but was included in this study to explore the effect of carbon chain extension).

Bartelt et al. (1992b) analyzed wind-tunnel responses of *C. hemipterus* to 25 tetraenes and one triene. Of the compounds tested with both species, **1**-**4** and **9** had significant attraction ($P < 0.001$) for *C. hemipterus* (1.3 ng with propyl acetate). For *C. humeralis*, **1** was the most attractive, but for *C. hemipterus*, **4** was the most active even though **1** was the most abundant in its pheromone blend. Compounds **5** and **6** were not tested for activity for *C. hemipterus* but **7**, which had good activity for *C. humeralis*, was unattractive to *C. hemipterus*. For *C. hemipterus*, **9** had attraction, but it was unattractive for *C. humeralis* at the same dose. Compounds **8**, **10**, **11**, and **12**, which had low activity in wind-tunnel bioassays for *C. humeralis*, were also poor attractants for *C. hemipterus*. For *C. humeralis*, **13** was the only triene attractive at the 1-ng level, but **13** had no activity for *C. hemipterus*.

Bartelt et al. (1990b) bioassayed trienes **13–17** at 10 ng doses against *C. freemani*. Compound **13**, a good attractant for *C. humeralis*, was both the most attractive for *C. freemani* and the most abundant in the pheromone blend, but any modifications to the structure reduced activity. Trienes **14** and **17** were also attractive for *C. freemani* but had <50% of the activity of **13**. Compounds **15** and **16** were not active for *C. freemani*, and **17** was not attractive for *C. humeralis*.

In the wind-tunnel bioassays, threshold doses for *C. humeralis* were often similar to those for other *Carpophilus* species responding to their own pheromones. Bartelt et al. (1993a) tested the two active pheromone components for *C. mutilatus* at 0.03 ng and found both significantly more attractive than the control. Petroski et al. (1994) found that *C. obsoletus* was significantly more attracted to 0.05 ng of its pheromone with propyl acetate than to propyl acetate alone.

Attraction of *C. freemani* to its own pheromone is due in part to the synergistic effect of the most active and abundant tetraene (**3**) and triene compound (**13**) (Bartelt et al., 1990b). However, no synergism for *C. humeralis* was observed when **1** and **13**, the most active tetraene and triene, were combined. In field trials, *C. humeralis* was captured at statistically equivalent levels in traps containing seven compounds (**1**, **2**, **3**, **4**, **13**, **14**, and **15**), four compounds (**1**, **2**, **3**, and **4**), or one compound (**4**) (Bartelt et al., 1995b). Extensive testing with *C. humeralis* would be required to absolutely rule out the possibility of synergism between some combination of components in Table 1.

In wind-tunnel bioassays, synergism between **1** and E, D, and P was dramatic. In general, adding any single compound from E, D, and P to **1** results in an increase in attraction. Binary combinations of any two of E, D, and P added to **1** also tended to increase the activity, but the ternary blend of E, D, and P plus **1** always gave the highest response. Further bioassays, testing various amounts of each chemical, may help define the individual importance of E, D, and P for *C. humeralis* attraction.

It is possible that the attraction of *C. humeralis* to so many different heterospecific pheromone components occurs because *C. humeralis* produces a similar pheromone blend, despite the lack of evidence for such a pheromone (Zilkowski et al., 1999). Another possibility is that because *C. humeralis* shares feeding sites with other *Carpophilus* species (Bartelt et al., 1992, 1994; Blumberg et al., 1993; James et al., 1993, 1994), it may use heterospecific pheromones as host-finding kairomones. Cane et al. (1990) reported that males of sympatric populations of sibling *Ips* used cross-attraction to pheromones among species to find suitable food substrates. Eventual identification of a pheromone from *C. humeralis* would lead to a better understanding of the responses of *C. humeralis* to the pheromone components of so many other *Carpophilus* species.

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SOURCES OF VARIATION IN CONCENTRATION AND COMPOSITION OF FOLIAR MONOTERPENES IN TAMARACK (*Larix laricina*) SEEDLINGS: ROLES OF NUTRIENT AVAILABILITY, TIME OF SEASON, AND PLANT ARCHITECTURE

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Abstract—The effects of foliage class, time of season, and nutrient availability on monoterpene composition and concentration were evaluated. In the first experiment, we compared foliar monoterpenes of long shoots versus short shoots, which differ in age and origin, and differences due to nutrient availability and time of season. Both the concentrations and compositions of these monoterpenes were higher in the foliage of the long shoots. The only exceptions were the concentration of Δ-3-carene and the composition of β-pinene. Within a foliage class, the concentrations of seven monoterpenes and the percentages of five monoterpenes changed over the season. Of these, almost all declined, with the exceptions of β-pinene and an unknown. Nutrient availability significantly affected the concentration or composition of some monoterpenes, but only to a slight extent. β-Pinene and the unknown increased with nutrient availability, whereas α-pinene and myrcene decreased. A more comprehensive analysis of four foliage classes on a separate cohort of trees showed that all monoterpenes, except sabinene and terpinolene, differed between foliage classes, but there were few general patterns. Overall, within-tree variation in tamarack monoterpenes related to foliage class and age appears more important than between-tree variation due to nutrient availability. These results suppport the view that products of mevalonic acid biosynthesis, such as terpenes, do not follow predictions of the carbon–nutrient balance and growth differentiation hypotheses. Our overall results show that individual hosts need to be considered as phytochemical mosaics when interpreting herbivore–plant interactions.

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Key Words—Terpenoids, monoterpenes, tamarack, *Larix laricina*, conifer, phytochemistry, variation.

INTRODUCTION

Secondary plant compounds have long been recognized as important components of plant defense against herbivores and pathogens. Terpenes are among the most widespread and important secondary plant compounds in conifers, and can exert toxic, deterrent, and antifeedant effects on insect and vertebrate herbivores, and inhibitory effects on microorganisms (Lewinsohn et al., 1993; Gershenzon, 1994b; Langenheim, 1994).

The composition and concentration of foliar terpenes are influenced by many factors and can vary within and among trees. Terpenoid chemistry may vary among trees due to environmental and genetic influences (Gershenzon and Croteau, 1991; Langenheim, 1994). Within an individual tree, terpenes may vary with crown position (Carisey and Bauce, 1997), seasonal changes (Gershenzon and Croteau, 1991; Doran and Bell, 1994; Goralka et al., 1996) differences between needle positions (Wallin and Raffa, 1998), and differences among foliage classes (Ohigashi et al., 1981; Gershenzon and Croteau, 1991). Knowledge of variation in foliar chemistry is important in predicting tree–herbivore interactions, since temporal and spatial avoidance are important means by which herbivores contend with plants containing high levels of defensive compounds (e.g., Wagner et al., 1979).

Among-plant differences are the most widely studied sources of variation in plant terpenoids. Among-plant variation may be the consequence of environmental factors, such as light (Gleizes et al., 1980; Gershenzon and Croteau, 1991), moisture availability (Yani et al., 1993; Doran and Bell, 1994), nutrients (Gershenzon and Croteau, 1991; Kainulainen et al., 1996), and genetics (Maffei, 1990; Langenheim, 1994; Doran and Bell, 1994; Goralka and Langenheim, 1995; Rafii et al., 1996). In general, absolute concentrations of terpenes are influenced largely by environmental factors, whereas the relative composition of terpenoid mixtures is usually under strong genetic control (Gershenzon and Croteau, 1991; Langenheim, 1994). Nutrient deficiencies generally lead to increased terpenoid concentration, while fertilization with N, P, or K leads to lower terpenoid concentration or has no effect (Gershenzon and Croteau, 1991). There are exceptions to this pattern, however (e.g., Bjorkman et al., 1991; McCullough and Kulman, 1991).

Within-plant changes in terpenoid chemistry during a season are common, although patterns of variation differ (e.g., von Rudloff, 1975; Hall and Langenheim, 1986a; Cates and Redak, 1988; Vince et al., 1994; Nerg et al., 1994; Zou and Cates, 1995). Reasons for seasonal differences are not well understood

but, in general, more terpenoids are present in recently expanded than in mature foliage (Gershenzon and Croteau, 1991). Changes in terpenoids over a season may be due to altered biosynthesis, transport, or degradation rates, losses due to volatilization or leaching, changes in primary metabolism rates, "dilution" in more mature foliage, or a combination of the above (Gershenzon and Croteau, 1991). Foliar terpenoids can decline through catabolism, and thus may represent a form of stored fixed carbon that can be reutilized for other functions (Croteau, 1988; Gershenzon, 1994a,b).

Difference between foliage classes is another important source of within-tree variation in conifers (e.g., Ohigashi et al., 1981; Hall and Langenheim, 1986b). Foliage classes are determined by the shoot type on which they are borne and, if applicable, their location on an individual shoot. Shoot types can be characterized by their location on the tree, type of development, or type of bud from which they arise (Kramer and Kozlowski, 1979). Variation among foliage classes may arise from processes similar to those causing seasonal variation, since foliage classes can differ in age. Foliage classes can also have different morphologies (Kramer and Kozlowski, 1979), sources of nutrients (Myre and Camiré, 1996), and origins (Kramer and Kozlowski, 1979; Myre and Camiré, 1996), which can further influence foliar terpenoids.

Tamaracks (*Larix laricina*) are deciduous conifers that occur in the Great Lakes States to New England, Canada, and Alaska (Gower and Richards, 1990). As in other conifers, terpenoids are predominant and important secondary compounds in tamarack (Stairs, 1967; Ohigashi et al., 1981; von Rudloff, 1987). Tamarack and other *Larix* species have several attributes as models for phytochemical studies. First, they allow for comparisons of different life histories strategies among closely related species. For example, *Larix* rely largely on terpenoids (mevalonic acid pathway) for defense, as do most gymnosperm trees (Wagner et al., 1979; Ohigashi et al., 1981), yet they share the deciduous life history habit with angiosperm trees, many of which rely on other carbon-based (e.g. shikimic acid pathway) defenses. Second, *Larix* combines several of the attributes emphasized by current resource allocation theories (Bryant et al., 1983; Coley et al., 1985), and thus can facilitate testing. For example, resource allocation theories predict that trees growing in low resource habitats will exhibit slow growth, long-lived foliage, late successional status, and carbon-based secondary chemistry. Tamaracks grow in very nutrient-poor areas (Tilton, 1977; Tyrell and Boerner, 1987; Gower and Richards, 1990) and have carbon-based secondary chemistry, yet are fast growing, have short-lived foliage, and are early successional. Third, *Larix* exhibits a unique growing pattern and possesses several foliage classes that allow intensive study of phytochemical dynamics.

Tamaracks exhibit a complex pattern of growth relative to most northern temperate tree species (Hallé et al., 1978; Kramer and Kozlowski, 1979). There are up to five shoot types, each bearing one or two classes of foliage (Powell,

1995). There are two types of short shoots (Remphrey and Powell, 1985; Powell, 1987, 1995), and possibly three types of long shoots (Remphrey and Powell, 1984; Powell, 1995). Short shoots are either sylleptic (neoformed) or proleptic (preformed) (Powell, 1987, 1995), whereas long shoots are usually nonsylleptic (partly neoformed/partly preformed) or sylleptic (Clausen and Kozlowski, 1967, 1970; Remphrey and Powell, 1984). Proleptic long shoots probably exist but are not common (Remphrey and Powell, 1984; Powell, 1995). In tamarack, proleptic short shoots and nonsylleptic long shoots are the most common and arise first (Clausen and Kozlowski, 1967, 1970). Growth of sylleptic shoots starts after growth of proleptic short shoots and nonsylleptic long shoot growth is near completion. They arise from the growing leader or first-order branches from new lateral axis apical meristems (Remphrey and Powell, 1985; Powell and Vescio, 1986; Powell, 1995). Presence of sylleptic shoots is influenced by genotype (Remphrey and Powell, 1985), vigor and length of the parent shoot (Remphrey and Powell, 1985; Powell and Vescio, 1986) and light (Yip and Powell, 1991).

Each foliage class depends on different sources of nutrients for growth (Clausen and Kozlowski, 1967; Myre and Camiré, 1996). Preformed needles (i.e., foliage of proleptic short shoots, and the proximal foliage of the nonsylleptic long shoots) are supported mainly by reserves from previous years (Clausen and Kozlowski, 1967; Myre and Camiré, 1996). Neoformed needles (i.e., apical axial foliage of the nonsylleptic long shoots, and foliage of the short and long sylleptic shoots) are supported by current nutrient uptake, current photosynthate, retranslocation from expanded or expanding leaves, or, if necessary, reserves (Clausen and Kozlowski, 1967; Myre and Camiré, 1996). Consequently, foliar nutrients, which can affect secondary chemistry (Bryant et al., 1983), differ among the foliage of these shoot types in Larix (Powell, 1998; Myre and Camiré, 1996).

This research was conducted to elucidate sources of variation in foliar terpenoid chemistry within and among larch seedlings.

METHODS AND MATERIALS

Effects of Time of Season and Nutrient Level on Foliar Monoterpenes of Long and Short Shoots. Two-year-old tamarack seedlings were bare-root lifted from the Minnesota Department of Natural Resources General Andrews Nursery in Willow River, Minnesota, and received on April 22, 1997. The seedlings were derived from seed collected from a tamarack plantation in Lake of the Woods County, Minnesota in 1987. They were placed in cold storage until planting in greenhouses on April 29, 1997.

The tamarack seedlings were planted in 18-liter pots in a medium of washed

silica sand and peat (16:1). They were exposed to ambient light until June, when the greenhouses were painted with a thin coat of shading compound to reduce heat. Temperatures in the greenhouse ranged from 19°C to 39°C during the period April 29–July 24, 1997. Plants were watered once or twice daily, as needed. The pH of the soil leachate ranged from 6.7 to 6.9.

Trees were fertilized by topdressing Osmocote 19-6-12 and incorporating Micromax and Sprint iron chealate into the top 15 cm of soil. The nutrient levels were chosen to represent the levels at which growth occurred with no apparent toxicity, the minimum level at which no mortality occurred, and a rate between the two (50.0 g, 0.5 g, and 10.0 g Osmocote 19-6-12, respectively), hereafter referred to as high, low, and medium nutrient availability. All trees were given equal amounts of micronutrients as 3 g of Micromax and 0.5 teaspoon of Sprint iron chealate.

Foliage from 15 randomly selected trees from each nutrient level was collected on May 21, June, 24, and July 24, 1997. Foliage of each tree was separated into proleptic short shoot foliage and nonsylleptic long shoot foliage. No sylleptic shoots were present. The foliage was frozen in liquid nitrogen and kept frozen until chemical analysis.

Data were analyzed by three-way split-plot ANOVA. Time of season and nutrient level were the whole plot factors, and foliage class was the split plot factor. All percentage data were transformed using the square root of the sum of the percentage and 0.5: $[\sqrt{(\% + 0.5)}]$. No transformations were necessary for the monoterpene concentration data. If a source of potential variation was shown to have a significant effect, the treatments were analyzed separately by two-way ANOVA (with split plot, if appropriate). If a source of potential variation had no significant effect, a two-way ANOVA (with split plot, if appropriate) was performed without that variable.

Effects of Foliage Class and Plant Architecture on Foliar Monoterpenes. Two-year-old tamarack seedlings were bare-root lifted from Great Lakes Nursery, Wausau, Wisconsin, in October 1996. The seedlings were grown from seed collected from a tamarack plantation in Baudette, Minnesota, in 1994. They were maintained in cold storage until planting on January 16, 1997, at the University of Wisconsin-Madison Biotron greenhouses. They were planted in 18-liter pots containing potting soil (Fafard mix 2). Supplemental light was supplied at 16L:8D. Temperature in the greenhouse was maintained at 24°C, and seedlings were watered with 500 ml at 8 AM and 250 ml at 4 PM every day. Trees were fertilized as described above, except each tree received 10 g of Osmocote.

Foliage from each of the 49 trees was collected on April 11, 1997 and was separated into proleptic short shoot foliage, proximal nonsylleptic long shoot foliage, distal nonsylleptic long shoot foliage, and sylleptic long shoot foliage. The proximal foliage was collected from the most proximal third of each nonsylleptic long shoot, and the distal foliage was collected from the most distal

third of each nonsylleptic long shoot. The foliage was frozen in liquid nitrogen and kept frozen until chemical analysis.

Data were analyzed by one-way ANOVA. All percentage data were transformed using the sum of the square root of the percentage and 0.5: $[\sqrt{(\% + 0.5)}]$. No transformations were necessary for the monoterpene concentration data.

Chemical Analysis. The concentration (milligrams per gram of foliage) and composition (percentage of total monoterpenes) of foliar monoterpenes were determined by GLC following the procedure of Raffa and Steffeck (1988). Foliage was finely chopped and extracted in 10 ml of 0.1% *p*-cymene, which does not occur in tamarack foliage, is easily separated from those monoterpenes present in the foliage, and served as an internal standard. After 24 hr, foliage was separated from the extract over vacuum filtration. The foliage and Buchner funnel were rinsed and the rinsate was added to the extract. The volume of the extract was brought to 10 ml by evaporating under nitrogen. A 1.5 ml portion of each extract was used for monoterpene analysis.

A Shimadzu GLC 14A (Shimadzu Scientific Instruments, Inc. Columbia, Maryland) was used for all monoterpene analysis. Separations were performed on a 25-m × 0.25-mm bonded fused silica open tubular polyethylene glycol column (Alltech Assoc., Deerfield, Illinois). Oven temperature was at 60°C for the first 10 min and was increased 10°C each minute for 10 min until a final temperature of 160°C was reached. Helium was the carrier gas, and flow was maintained at 30 cm/sec.

Concentrations were determined by comparing integrated peak areas (Shimadzu C-R3A Chromatopac) with known standard equations. Standard equations were calculated by regressing peak areas of synthetic monoterpenes on the corresponding known amount. Each standard curve generated an r^2 value of at least 0.998 (Powell, 1998). β-Phellandrene and unknown 1 were quantified by comparing their percentage of the total with the percentage of a known amount of internal standard. All monoterpene concentrations were calculated as milligrams of monoterpene per gram of fresh foliage.

RESULTS

Effects of Time of Season and Nutrient Level on Foliar Monoterpenes of Long and Short Shoots

Concentration. Sources of variation in concentrations of monoterpenes in tamarack foliage are shown in Table 1. There were several treatment effects due to time of season and foliage class and several foliage × season interactions. There were fewer effects due to nutrient level. For each monoterpene, the whole plot error (due to nutrient level and time of season) was larger than the split-plot error (due to foliage class).

TABLE 1. SOURCES OF VARIATION IN TAMARACK FOLIAR MONOTERPENE CONCENTRATION (mg/g FOLIAGE)[a]

Source	df	α-Pinene		β-Phellandrene		β-Pinene		Camphene		Δ-3-Carene	
		F	P	F	P	F	P	F	P	F	P
Season	2	15.19	<0.001	1.64	0.198	8.89	<0.001	8.65	<0.001	0.86	<0.424
Nutrients	2	0.15	0.862	0.96	0.385	8.39	<0.001	0.05	0.954	0.05	0.951
Season × Nutrients	4	1.22	0.307	1.52	0.199	2.85	0.027	0.89	0.475	0.72	0.579
Whole plot error	125	MSE = 1.1098		MSE = 0.0026		MSE = 0.9494		MSE = 0.1379		MSE = 0.0614	
Foliage	1	67.47	<0.001	6.75	0.011	5.75	0.018	78.71	<0.001	68.15	<0.001
Season × Foliage	2	2.16	0.119	6.52	0.002	22.65	<0.001	8.15	<0.001	6.05	0.003
Nutrients × Foliage	2	0.50	0.609	0.15	0.859	1.50	0.227	0.12	0.889	1.70	0.187
Seas × Nut × Fol	4	0.27	0.898	0.65	0.630	0.94	0.442	0.57	0.687	2.49	0.047
Split plot error	120	MSE = 0.6037		MSE = 0.0014		MSE = 0.6178		MSE = 0.0646		MSE = 0.0163	

Source	Limonene		Myrcene		Sabinene		Terpinolene		Unknown		Total	
	F	P	F	P	F	P	F	P	F	P	F	P
Season	1.02	0.3620	8.60	<0.001	21.12	<0.001	5.95	0.003	14.27	<0.001	9.74	<0.001
Nutrients	0.67	0.5115	2.03	0.135	0.85	0.428	1.05	0.352	6.62	0.002	2.06	0.131
Season × Nutrients	0.59	0.6740	1.09	0.367	0.70	0.596	1.37	0.246	4.07	0.004	2.03	0.094
Whole plot error	MSE = 0.0213		MSE = 0.0011		MSE = 0.0062		MSE = 0.0026		MSE = 0.0003		MSE = 5.349	
Foliage	52.27	0.0001	11.82	<0.001	22.26	<0.001	0.01	0.909	2.11	0.149	29.14	<0.001
Season × Foliage	6.65	0.0018	9.81	<0.001	1.60	0.206	9.73	<0.001	1.20	0.305	9.94	<0.001
Nutrients × Foliage	2.12	0.1248	0.29	0.752	1.67	0.193	0.78	0.463	4.38	0.015	0.92	0.402
Seas × Nut × Fol	1.76	0.1411	0.24	0.916	1.03	0.397	3.23	0.015	3.08	0.019	0.53	0.712
Split plot error	MSE = 0.0024		MSE = 0.0004		MSE = 0.0049		MSE = 0.0011		MSE = 0.0002		MSE = 3.554	

[a]Type III sums of squares (ANOVA).

All monoterpenes varied among sampling periods, except β-phellandrene, Δ-3-carene, and limonene (Table 1). β-Phellandrene, β-pinene, camphene, Δ-3-carene, limonene, myrcene, terpinolene, and total monoterpene concentration had significant season × foliage interactions (Table 1). Most monoterpene concentrations decreased over the season. This pattern occurred in both foliage classes (Figure 1). β-Pinene decreased in the short shoot foliage of the lowest nutrient level, but increased in the long shoot foliage of the highest nutrient level (Figure 1c). Unknown 1 did not appear until the second sampling period, and increased in the third sampling period (Figure 1j). When monoterpene concentrations of each foliage class were analyzed separately, there was a greater seasonal effect on the long shoot foliage than on the short shoot foliage (Table 2). There was no effect due to time of season in the short shoot foliage for β-pinene or camphene (Table 2).

Nutrient availability affected the overall concentrations of β-pinene and unknown 1 (Table 1). The concentrations of both β-pinene and unknown 1 were influenced by a nutrient × season interaction, and unknown 1 was affected by a nutrient × foliage interaction (Table 1). Among those monoterpenes affected by nutrient level, concentrations increased with increasing nutrients. β-pinene increased with increasing nutrient availability in both foliage classes in the last sampling period (Figure 1c). The concentration of unknown 1 increased with increasing nutrient availability only in the short shoot foliage in the last sampling period (Figure 1j).

Foliage class was a significant source of variation in total monoterpenes and all individual monoterpenes, except terpinolene and unknown 1 (Table 1). Most monoterpene concentrations were significantly higher in the long shoot foliage than in the short shoot foliage (Figure 1). The only exception was Δ-3-carene. When each sampling period was analyzed separately, differences between foliage classes occurred more frequently in the early-season sampling period than in the late-season sampling period (Table 3). Differences in total monoterpenes among foliage classes, however, occurred only in the mid- and late-season sampling periods (Table 3).

In general, tamarack growth increased with increasing nutrient level (Table 4). There were, however, no strong correlations between diameter or height growth and concentration of monoterpenes. Of the monoterpenes present, only the concentrations of α-pinene, β-pinene, and Δ-3-carene were related to growth, and these relationships were weak, with r^2 values less than 0.315 (Powell 1998).

Composition. With few exceptions, monoterpene composition (percentage of total monoterpenes) followed trends similar to monoterpene concentration (Figure 2). For all monoterpenes except β-pinene, the whole plot error (due to nutrient level and time of season) was larger than or equal to the split-plot error (due to foliage class) (Powell, 1998).

The percentages of α-pinene, β-pinene, sabinene, terpinolene, and unknown

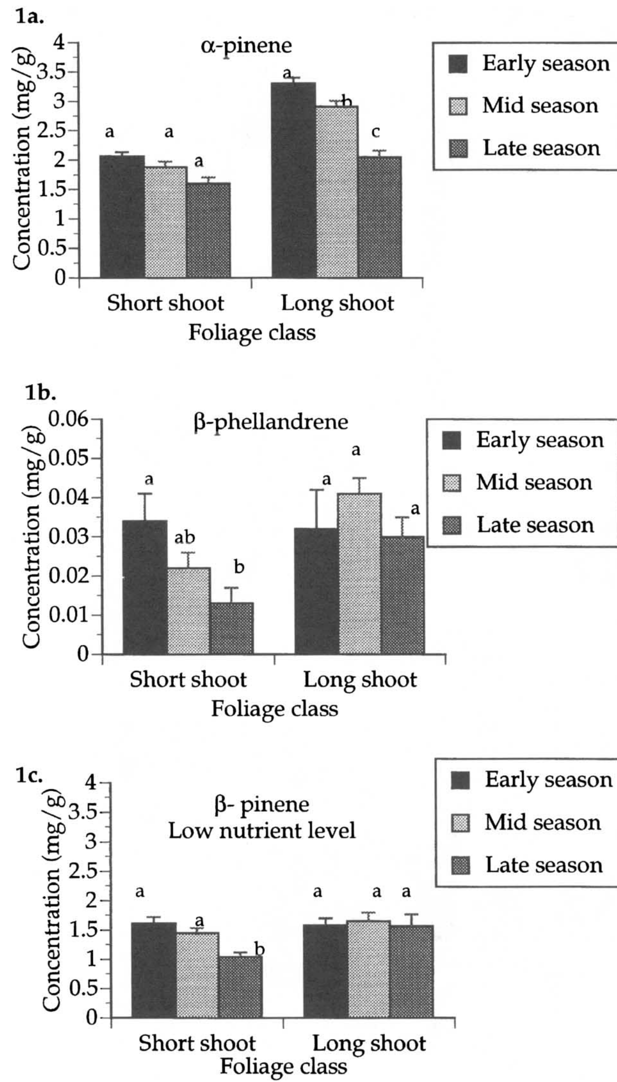

FIG. 1. Effects of foliage class, time of season, and nutrient availability on the concentration (mg monoterpene/g wet weight foliage) of foliar monoterpenes in tamarack. Standard error bars are given for each mean. Means, within a foliage class, followed by the same letter are not significantly different at $P < 0.05$ (Fisher's protected LSD).

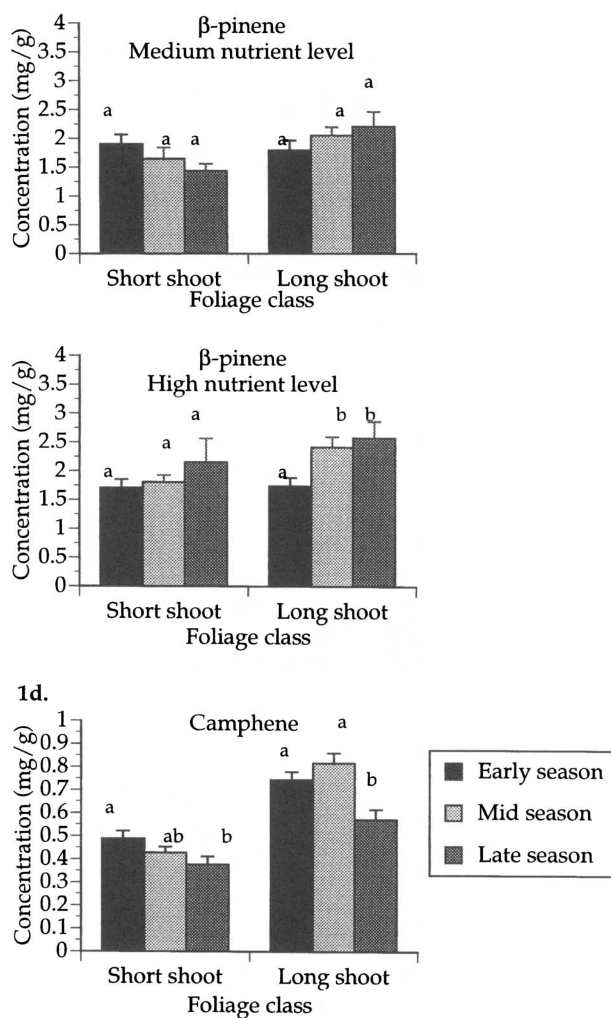

FIG. 1. CONTINUED.

1 were affected by time of season (F = 3.92–30.71; df = 2, 125; P < 0.001–0.0224. Season did not affect camphene or myrcene. The percentage of all affected monoterpenes except β-pinene and unknown 1 was least in the late sampling period (Figure 2).

The percentages of α-pinene, β-pinene, myrcene, and unknown 1 were affected by nutrient availability (F = 3.24–15.76; P < 0.001–0.0424; df = 2, 125). The percentages of α-pinene and myrcene decreased with nutrient avail-

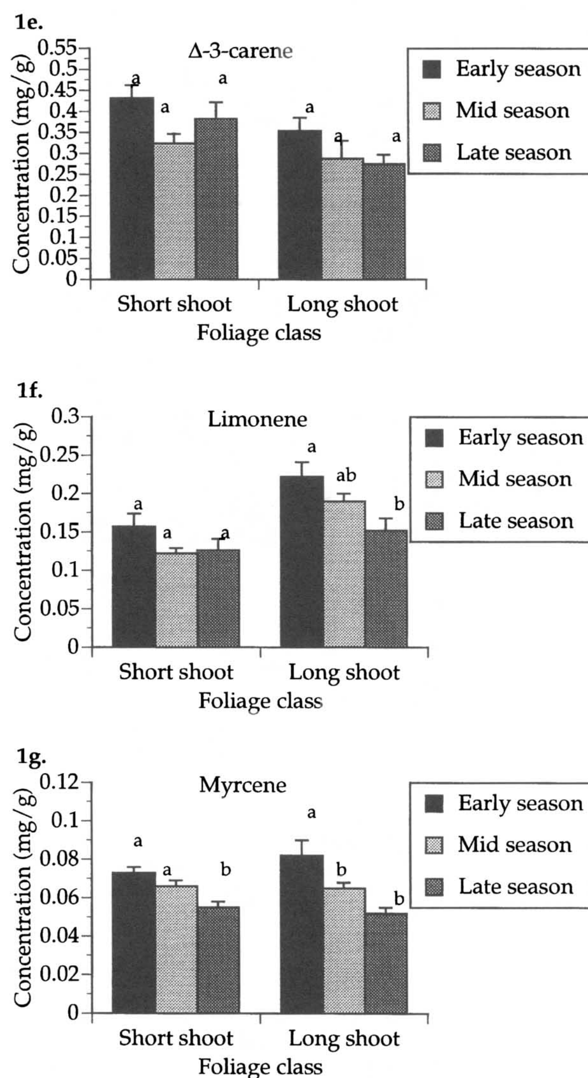

FIG. 1. CONTINUED.

ability, whereas the percentages of β-pinene and unknown 1 increased with nutrient availability (Figure 2).

Foliage class affected the percentages of all monoterpenes except β-phellandrene and terpinolene ($F = 4.30$–192.56; $P < 0.001$–0.0424; $df = 1, 120$). These results were unlike those of monoterpene concentration, where there was

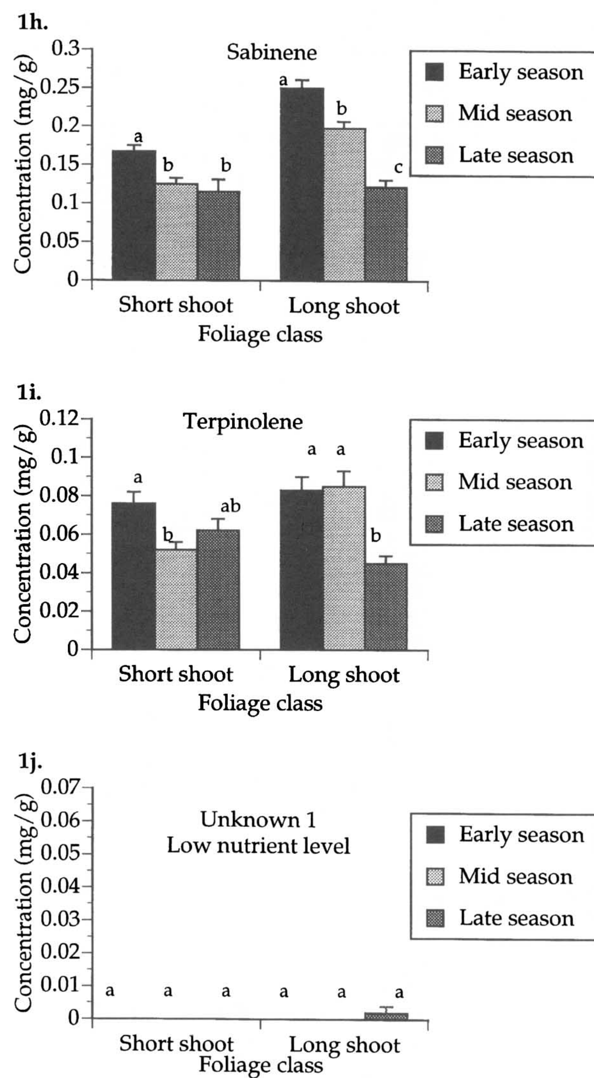

FIG. 1. CONTINUED.

no difference among foliage classes for terpinolene and unknown 1. β-Pinene was the only monoterpene whose percentage was significantly higher in the short shoot foliage than in the long shoot foliage ($F = 55.40$; $P < 0.001$; $df = 1, 20$).

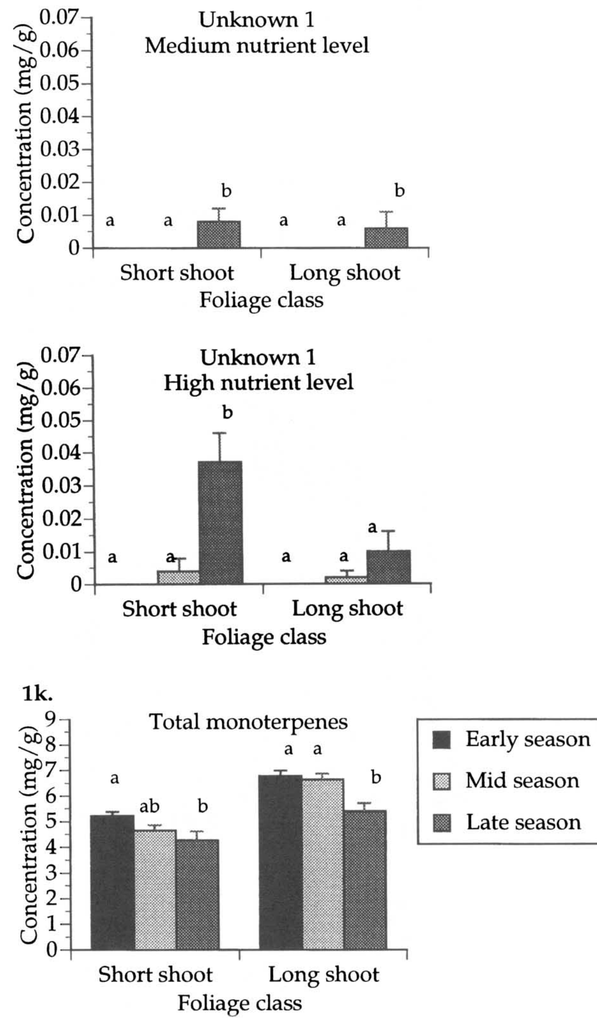

FIG. 1. CONTINUED.

Effects of Foliage Class and Plant Architecture on Foliar Monoterpenes

Concentration. Foliage class affected all monoterpenes except sabinene and terpinolene (Table 5). Five monoterpenes differed in concentration between the proleptic short shoots and the nonsylleptic long shoots. There was more β-pinene and Δ-3-carene in the short shoot foliage than in the proximal or distal foliage of the nonsylleptic long shoots. Conversely, there was less β-phellandrene, camphene,

TABLE 2. SOURCES OF VARIATION, PARTITIONED BY FOLIAGE CLASS, IN TAMARACK FOLIAR MONOTERPENE CONCENTRATION (mg/g FOLIAGE)[a]

			α-Pinene		β-Phellandrene		β-Pinene		Camphene		Δ-3-Carene	
Foliage	Source	*df*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Short shoot	Season	2	5.62	0.005	4.00	0.021	0.77	0.465	2.87	0.061	2.75	0.068
	Nutrients	2	1.17	0.315	0.57	0.569	5.50	0.005	0.47	0.626	0.58	0.564
	Seas × Nut	4	1.74	0.145	0.53	0.711	2.20	0.073	2.01	0.097	1.06	0.381
Long shoot	Season	2	35.77	<0.001	0.67	0.513	3.92	0.022	8.83	<0.001	1.92	0.152
	Nutrients	2	0.97	0.384	0.63	0.535	8.35	<0.001	0.06	0.945	1.33	0.269
	Seas × Nut	4	0.22	0.925	1.71	0.151	1.30	0.274	0.03	0.998	0.88	0.479

		Limonene		Myrcene		Sabinene		Terpinolene[b]		Unknown		Total	
Foliage	Source	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Short shoot	Season	1.76	0.176	7.94	<0.001	5.81	0.004			15.77	<0.001	3.53	0.033
	Nutrients	0.93	0.398	1.13	0.327	0.36	0.700			11.55	<0.001	2.69	0.072
	Seas × Nut	0.87	0.483	1.71	0.152	0.43	0.784			8.96	<0.001	2.37	0.056
Long shoot	Season	4.29	0.016	7.59	<0.001	45.09	<0.001			5.02	0.008	8.63	<0.001
	Nutrients	0.81	0.447	1.00	0.369	3.63	0.029			1.36	0.267	3.60	0.030
	Seas × Nut	0.58	0.679	0.46	0.766	0.28	0.894			0.75	0.560	0.36	0.837

[a] Type III sums of squares (ANOVA).
[b] Blanks indicate no significance between partitioned treatments.

TABLE 3. SOURCES OF VARIATION, PARTITIONED BY TIME OF SEASON, IN TAMARACK FOLIAR MONOTERPENE CONCENTRATION (mg/g FOLIAGE)[a]

Season	Source	df	α-Pinene		β-Phellandrene[b]		β-Pinene		Camphene		Δ-3-Carene[b]	
			F	P	F	P	F	P	F	P	F	P
Early	Foliage	1	14.88	<0.001			52.89	<0.001	6.21	0.017		
	Nutrients	2	1.07	0.353			0.00	0.995	1.57	0.220		
	Fol × Nut	2	1.09	0.347			1.69	0.198	0.39	0.677		
Mid	Foliage	1	38.44	<0.001			2.19	0.147	55.18	<0.001		
	Nutrients	2	0.04	0.965			1.86	0.169	0.03	0.971		
	Fol × Nut	2	0.03	0.970			1.38	0.264	0.38	0.683		
Late	Foliage	1	19.77	<0.001			17.21	<0.001	27.24	<0.001		
	Nutrients	2	1.83	0.173			10.31	<0.001	0.74	0.484		
	Fol × Nut	2	0.21	0.808			1.02	0.372	0.43	0.652		

Season	Source	Limonene[b]		Myrcene		Sabinene		Terpinolene		Unknown		Total	
		F	P	F	P	F	P	F	P	F	P	F	P
Early	Foliage			41.17	<0.001	6.79	0.013	5.54	0.024	c	c	0.28	0.602
	Nutrients			1.30	0.283	0.00	1.000	0.02	0.982	c	c	0.47	0.631
	Fol × Nut			0.81	0.453	4.77	0.014	1.46	0.244	c	c	1.32	0.278
Mid	Foliage			8.70	0.005	28.21	<0.001	9.22	0.004	1.09	0.303	22.24	<0.001
	Nutrients			2.73	0.077	0.47	0.626	0.07	0.932	1.08	0.351	0.37	0.695
	Fol × Nut			0.26	0.776	0.01	0.995	1.13	0.332	1.08	0.351	0.31	0.734
Late	Foliage			2.20	0.146	2.97	0.092	2.50	0.122	1.54	0.222	17.82	<0.001
	Nutrients			0.03	0.969	1.52	0.230	6.31	0.004	6.27	0.004	4.88	0.012
	Fol × Nut			0.07	0.931	0.96	0.391	5.70	0.007	3.86	0.029	0.63	0.540

[a]Type III sums of squares (ANOVA).
[b]Blanks indicate no significance between partitioned treatments.
[c]Absence of compound.

TABLE 4. EFFECTS OF NUTRIENT AVAILABILITY ON DIAMETER AND HEIGHT GROWTH IN TAMARACK SEEDLINGS[a]

Time of season	Nutrient level	N	Increase in diameter (cm, mean ± SE)	N	Increase in height (cm, mean ± SE)
Early	Low	219	0.1 ± 0.0 a	219	0.0 ± 0.0
	Medium	227	0.1 ± 0.0 a	227	0.0 ± 0.0
	High	221	0.0 ± 0.0 b	221	0.0 ± 0.0
Mid	Low	175	0.4 ± 0.0 a	194	3.7 ± 0.1 a
	Medium	207	0.8 ± 0.0 b	208	4.6 ± 0.1 b
	High	187	1.0 ± 0.0 c	194	4.4 ± 0.1 b
Late	Low	15	0.6 ± 0.1 a	56	1.4 ± 0.2 a
	Medium	13	1.0 ± 0.1 ab	79	4.6 ± 0.4 b
	High	15	1.4 ± 0.2 b	110	9.2 ± 0.6 c

[a]Means, within a collection period, followed by different lower case letters are significantly different at $P < 0.05$ (Fisher's protected LSD).

and limonene in the short shoot foliage than in the proximal or distal foliage of the nonsylleptic long shoots. There was less α-pinene in the short shoot foliage than in the proximal foliage of the nonsylleptic long shoots, and more myrcene in the short shoot foliage than in the proximal foliage of the nonsylleptic long shoot (Table 5).

The concentrations of α-pinene, camphene, and total monoterpenes differed between the sylleptic long shoots and the nonsylleptic long shoots. In all cases, the sylleptic long shoot foliage contained significantly higher concentrations than the nonsylleptic long shoot foliage (Table 5).

There were several differences in monoterpene concentration between the short shoots and the sylleptic shoots. The concentrations of α-pinene, camphene, and limonene were significantly higher in the sylleptic long shoots than in the short shoots. Conversely, the concentrations of Δ-3-carene and myrcene were significantly higher in the short shoot foliage than the sylleptic long shoot foliage (Table 5).

There was no difference in any monoterpene between the proximal and distal foliage of the non-sylleptic long shoots (Table 5).

Composition. As with monoterpene concentration, the percentages of all monoterpenes, except sabinene and terpinolene, were affected by foliage class (Table 6). There were, however, some differences in trends between monoterpene concentration and composition. The percentages of α-pinene and camphene were significantly lower in the short shoot foliage than all other foliage classes. The percentage of β-pinene in the sylleptic long shoot foliage was significantly lower than the percentage in the short shoot or nonsylleptic long shoot foliage. The percentage of myrcene in the proximal long shoot foliage was significantly higher than in the sylleptic shoot foliage (Table 6).

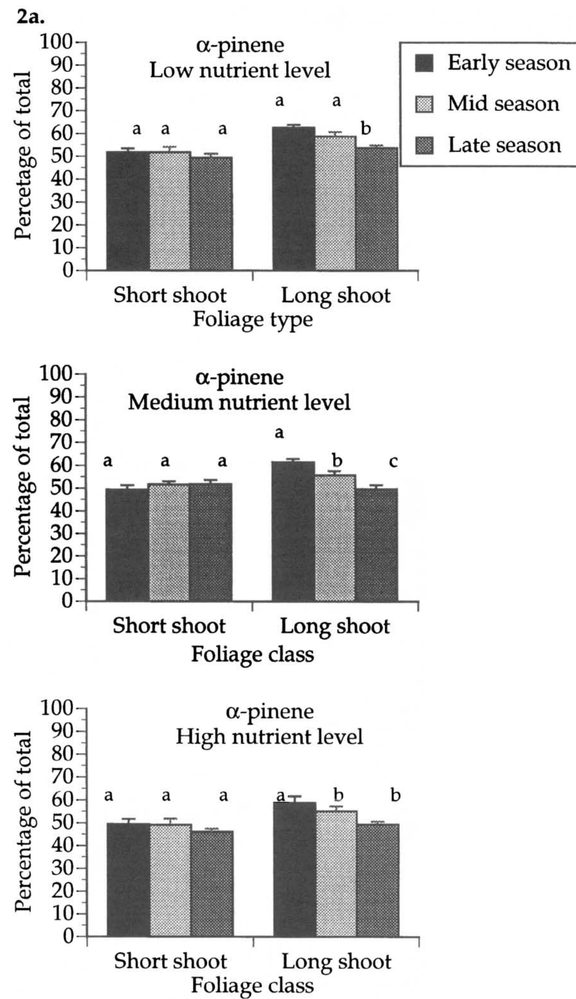

FIG. 2. Effects of foliage class, time of season, and nutrient availability on the concentration (percentage of total) of foliar monoterpenes in tamarack. Standard error bars are given for each mean. Means, within a foliage class, followed by the same letter are not significantly different at $P < 0.05$ (Fisher's protected LSD).

DISCUSSION

Our results show that variation in terpenoids in tamarack foliage can occur within and among individuals of the same population and that this variation depends on the age and class of foliage, and, to a lesser extent, nutrient availabil-

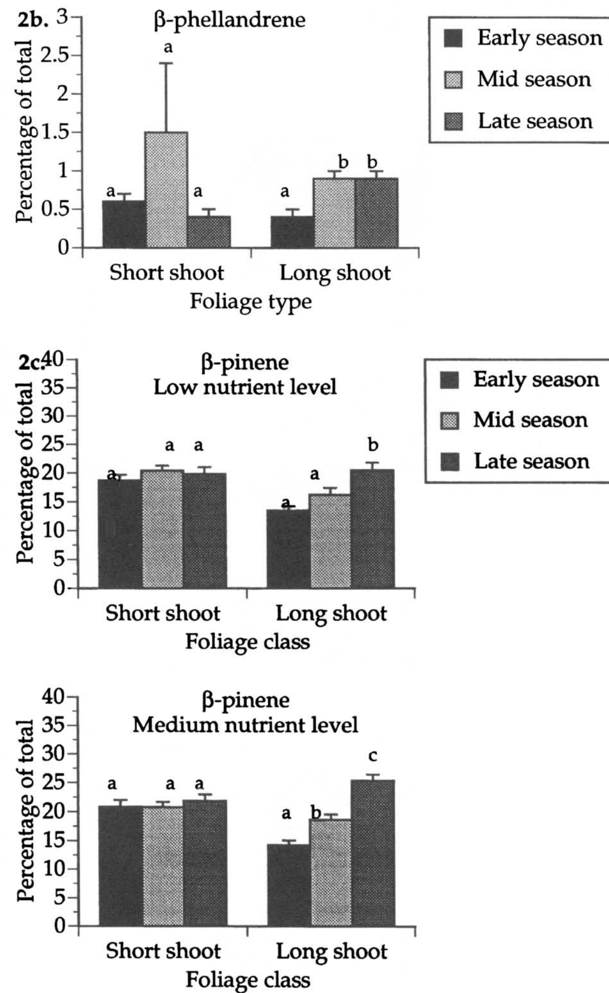

FIG. 2. CONTINUED.

ity. These results agree with previous reports showing within-tree variation in foliar monoterpenes of conifers (e.g., Ohigashi et al., 1981; Cates, 1996; Wallin and Raffa, 1998).

The concentration of total monoterpenes, the concentrations of 6 of the 10 monoterpenes, and the percentages of 5 of the 10 monoterpenes, varied over a season. Where seasonal effects were significant, all except two monoterpenes decreased with time. Several processes could contribute to decreases

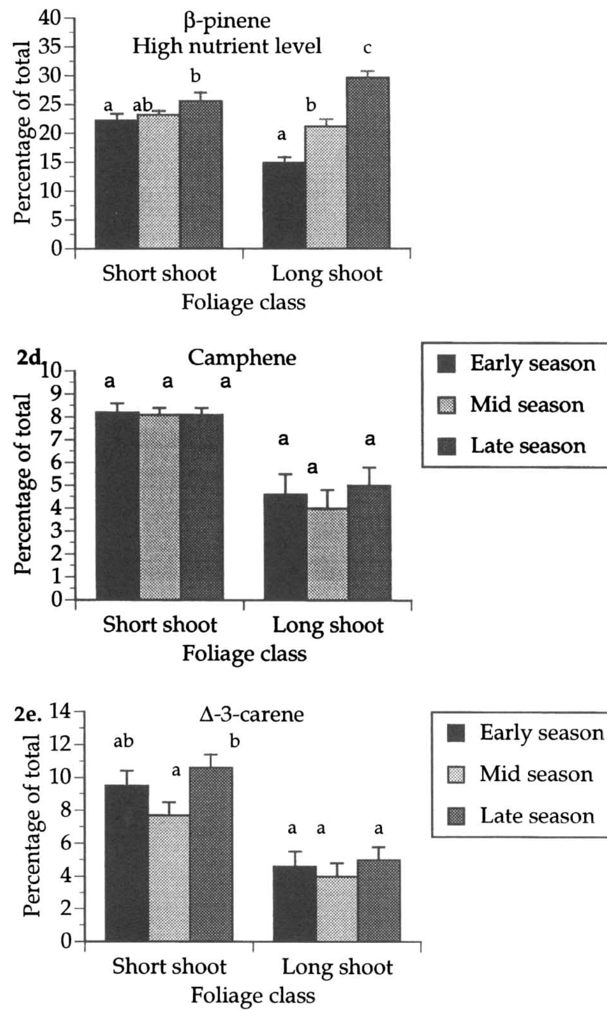

FIG. 2. CONTINUED.

in terpene concentrations in conifer foliage over a season (Gershenzon and Croteau, 1991; Gershenzon, 1994b). "Dilution" through growth (Gershenzon and Croteau, 1991) seems unlikely in our system, since the foliage of the short shoots was fully expanded on all sampling dates. Catabolism, in which monoterpenes are degraded, and the products of degradation are recycled back into primary metabolism, has been confirmed in only a few systems (Croteau, 1988; Gershenzon, 1994a). However, decreases in monoterpenes in vegetative organs late

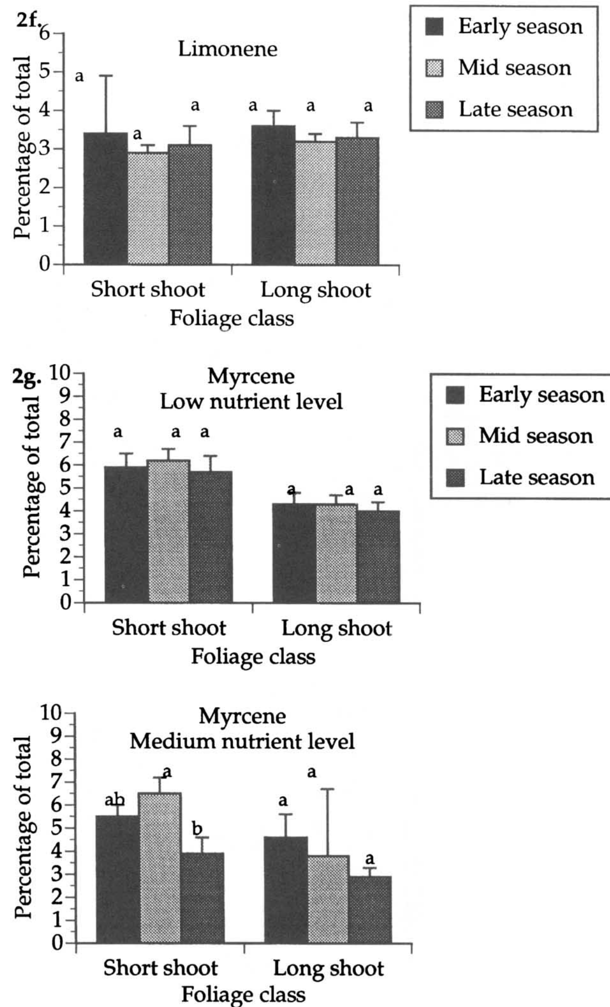

FIG. 2. CONTINUED.

in development, which may be indicative of catabolism (Gershenzon, 1994b), have been seen in several systems (Hall and Langenheim, 1986a; Svoboda et al., 1990; Dudai et al., 1992). The seasonal decline in most individual, and total, monoterpenes observed here indicates that catabolism may occur in tamarack seedlings. However, volatilization, leaching, metabolic turnover, and transport of monoterpenes to other plant parts cannot be ruled out as sources of the decline (Gershenzon, 1994a,b).

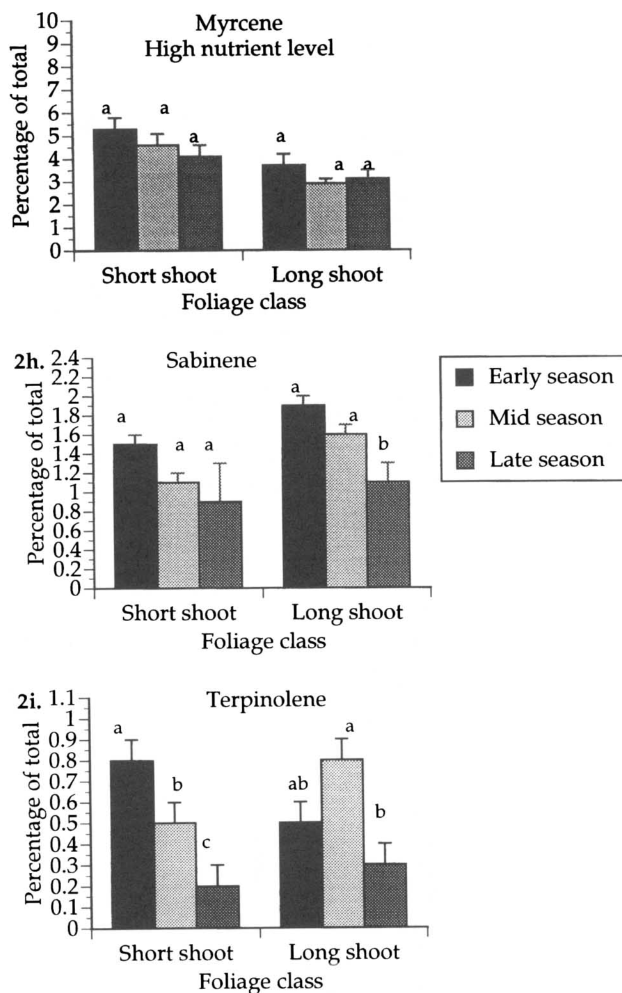

FIG. 2. CONTINUED.

The complexity of *Larix* architecture and foliar ontogeny contributed greatly to within plant phyochemical variation. The concentrations and percentages of most individual and total monoterpenes differed between the two foliage classes in the first experiment, and among the four foliage classes in the second experiment. Factors that contributed to these differences include different origins (preformed vs. neoformed), ages (short > proximal long > distal long ≥ sylleptic long), and stages of growth (short and nonsylleptic long shoots had set bud at

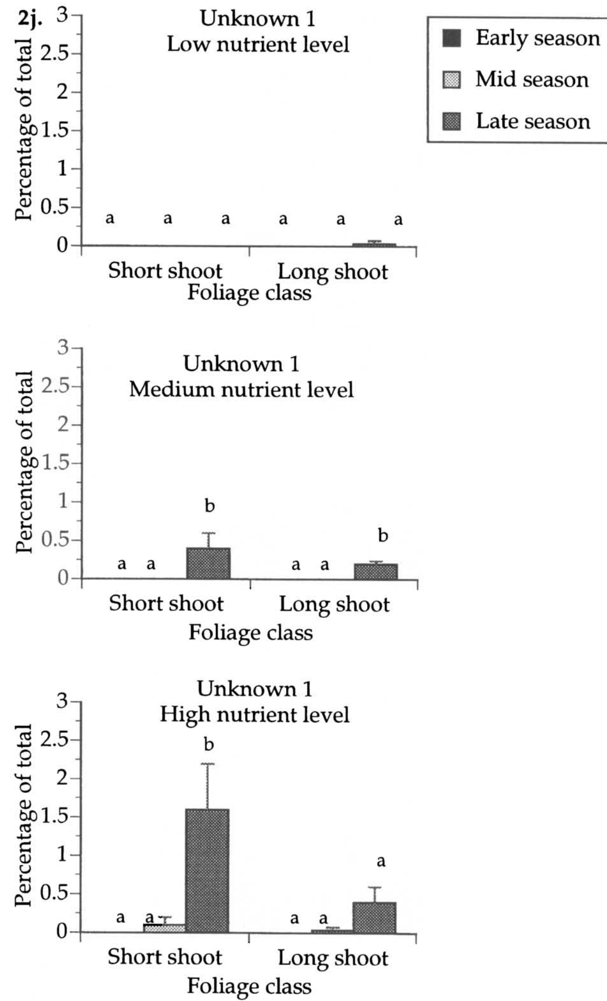

FIG. 2. CONTINUED.

time of collection, sylleptic long shoots had not). There were, however, several differences between the results of the first and second experiments. For example, in the first experiment, concentrations of most monoterpenes were higher in the nonsylleptic long shoot foliage than in the proleptic short shoot foliage, but in the second experiment, less than half of the monoterpenes were present in higher amounts in the foliage of the nonsylleptic long shoot than in the foliage of the proleptic short shoot foliage or the other foliage classes. Some possible

TABLE 5. EFFECT OF FOLIAGE CLASS[a] ON MONOTERPENE CONCENTRATION (mg MONOTERPENE/g FOLIAGE)[b]

	Proleptic short	Nonsylleptic Long proximal	Nonsylleptic Long distal	Sylleptic long	F	P
α-Pinene	2.11 (0.12) a	2.61 (0.13) b	2.33 (0.11) ab	3.21 (0.17) c	12.88	<0.001
β-Phellandrene	0.02 (0.01) a	0.04 (0.01) b	0.04 (0.00) b	0.03 (0.00) ab	3.05	0.030
β-Pinene	2.46 (0.16) a	1.96 (0.11) b	1.99 (0.10) b	2.15 (0.11) ab	3.65	0.014
Camphene	0.48 (0.04) a	0.87 (0.05) b	0.76 (0.04) b	1.04 (0.05) c	27.67	<0.001
Δ-3-Carene	1.26 (0.09) a	0.67 (0.08) b	0.58 (0.07) b	0.67 (0.06) b	16.77	<0.001
Limonene	0.11 (0.01) a	0.17 (0.01) b	0.17 (0.01) b	0.20 (0.01) b	10.31	<0.001
Myrcene	1.00 (0.09) a	0.73 (0.06) b	0.84 (0.07) ab	0.70 (0.05) b	3.85	0.011
Sabinene	0.21 (0.00) a	0.23 (0.01) a	0.19 (0.01) a	0.28 (0.02) a	2.07	0.105
Terpinolene	0.14 (0.02) a	0.14 (0.01) a	0.11 (0.01) a	0.15 (0.01) a	1.86	0.139
Total	7.79 (0.36) ab	7.42 (0.30) a	7.00 (0.27) a	8.42 (0.34) b	3.61	0.015

[a]Proleptic short, nonsylleptic long proximal: preformed, growth complete; nonsylleptic long distal: neoformed, growth complete; sylleptic long shoot foliage: neoformed, growth not complete.
[b]Means, within one monoterpene, followed by the same letter, are not significantly different at $P < 0.05$ (Fisher's protected LSD; type III sums of squares); $df = 3$.

reasons for differences between experiments include environmental factors (soil types, nutrients, temperature, lighting), intervals after planting at which foliage was collected, and seed sources.

The effects of nutrient availability on terpenoid content were subtle. Total

TABLE 6. EFFECT OF FOLIAGE CLASS[a] ON MONOTERPENE COMPOSITION (PERCENTAGE OF TOTAL)[b]

	Proleptic short	Nonsylleptic Long proximal	Nonsylleptic Long distal	Sylleptic long	F	P
α-Pinene	33.21 (1.03) a	45.79 (1.15) b	47.17 (1.40) b	48.47 (1.52) b	29.97	<0.001
β-Phellandrene	0.58 (0.14) a	1.02 (0.08) b	0.94 (0.15) b	0.74 (0.10) ab	3.02	0.031
β-Pinene	22.83 (1.08) a	19.93 (0.50) b	18.29 (0.58) bc	16.53 (0.70) c	10.49	<0.001
Camphene	6.41 (0.78) a	10.52 (0.39) b	10.91 (0.41) b	12.57 (1.41) b	19.4	<0.001
Δ-3-Carene	24.88 (1.46) a	11.12 (1.31) a	12.02 (1.42) a	11.03 (1.33) a	24.04	<0.001
Limonene	1.52 (0.22) a	2.92 (0.22) b	2.74 (0.12) b	2.58 (0.14) b	13.04	<0.001
Myrcene	5.91 (1.07) a	5.63 (0.60) a	4.33 (0.63) ab	2.96 (0.24) b	4.21	0.007
Sabinene	1.95 (0.64) a	1.61 (0.10) a	1.85 (0.11) a	2.45 (0.48) a	0.78	0.504
Terpinolene	0.73 (1.13) a	1.47 (0.13) a	1.74 (0.13) a	3.08 (1.45) a	0.56	0.642

[a]Terminology as in Table 5.
[b]Means, within one monoterpene, followed by the same letter, are not significantly different at $P < 0.05$ (Fisher's protected LSD; type III sums of squares); $df = 3$.

monoterpenes did not change due to nutrient availability and concentrations of only two monoterpenes and the percentages of only four monoterpenes were affected. It appears, at least within one growing season, that different nutrient availabilities have little or no effect on monoterpene content in tamarack seedlings. Resource allocation theories predict that carbon-based secondary compounds, such as monoterpenes, will decrease with increasing nutrient availabilities due to an excess in carbon (Bryant et al., 1983; Coley et al., 1985; Herms and Mattson, 1992). It has been suggested, however, that products of the mevalonic acid pathway, such as terpenoids, respond less to resource availability than do products of the shikimic acid pathway (Reichardt et al., 1991; Koricheva et al., 1998). Our results support this modification of resource allocation theories.

The extent to which the patterns observed here can be extrapolated to sources of within-plant variation in other systems cannot be established without additional information. However, the overall conclusion that folivores attempting to avoid or track secondary plant compounds must contend with a highly variable resource, and that these patterns are more complex than can be described by simple seasonal or resource-based trends, seems likely to apply to many systems. In addition, our results support the view that deciduous conifers contain mixtures of attributes normally restricted along taxonomic lines in temperate perennial plants, and thus can contribute to our overall understanding of plant–insect interactions.

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INTRASPECIFIC CHEMICAL RECOGNITION IN THE LIZARD *Liolaemus tenuis*

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Abstract—Experimental tests were conducted to determine whether females and males of the tree-dwelling lizard *Liolaemus tenuis* (Tropiduridae) show intraspecific chemical recognition during breeding and postreproductive seasons. Animals were individually maintained in plastic enclosures for one week. Thereafter, the number of tongue-flicks that a lizard performed in the enclosure of a male, a female, its own home enclosure, and a control (unused) enclosure were recorded. In both seasons, males and females made fewer tongue-flicks in their home enclosures than in any other one, indicating a recognition of a familiar place, probably a chemical self-recognition. Conspecific chemical recognition was season dependent. During the post-reproductive season, lizards tongue-flicked at similar rates in conspecific and control enclosures, while during the breeding season enclosures of females elicited more tongue-flicks by both sexes, and the overall tongue-flick rates were higher than in the postreproductive season. Results are discussed within the context of the social system of the species.

Key Words—*Liolaemus* lizards, conspecific chemical recognition, tongue-flick.

INTRODUCTION

In recent years it has become clear that reptiles depend on more than vision for their orientation and communication. The importance of chemoreception for gathering information from the environment is now widely recognized (for reviews see Halpern, 1992; Mason, 1992). To sample the environment, squamates protrude their tongues to obtain chemical samples by touching either a

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substrate or the air, and then deliver these samples to the vomeronasal organ (Halpern, 1992). Much of our knowledge of squamate chemoreception and correlated behavioral phenomena pertains to snakes, which possess highly sensitive vomeronasal organs and utilize chemical stimuli in diverse ecological situations. Nevertheless, chemoreception is also widespread in lizards (Halpern, 1992; Mason, 1992; Cooper, 1994), although its use varies among lizard families (for reviews see Mason, 1992; Cooper, 1994). Because *Liolaemus* lizards (Tropiduridae) are sit-and-wait foragers, they would not be expected to detect prey chemicals (De Perno and Cooper, 1995; Cooper, 1994; 1995). However, no studies have been published of conspecific chemical recognition in any of the more than 150 species in this genus (Etheridge, 1995), nor in any other tropidurid.

Chemical secretions used in intraspecific communication may come from specific glands, such as urodaeal (Cooper and Trauth, 1992) and femoral glands (Alberts, 1993), from the body surface (Mason and Gutzke, 1990; Steele and Cooper, 1997), or from feces (Werner et al., 1987). One diagnostic characteristic of Tropiduridae is the presence of precloacal or preanal pores (Frost and Etheridge, 1989), which in most *Liolaemus* are only present in males (Donoso-Barros, 1966). These pores have been described as similar to sebaceous glands and more active during the breeding season (Donoso-Barros, 1966), when secretions appear enlarged and more reddish (A. Labra, personal observations). In gekkonid lizards these pores are openings of precloacal glands that produce holocrine secretions (Maderson, 1972), and in amphisbaenians these pores are associated with pheromonal secretions (Cooper et al., 1994). Therefore, even though it is not clear whether precloacal pores of *Liolaemus* are homologous with those described for other species, pheromone secretion is a distinct possibility.

The tree-dwelling *Liolaemus tenuis* is highly sexually dimorphic, males being more colorful and larger than females. The species has a polygynous mating system; males are territorial and females establish hierachies based on agonistic behaviors inside a territory (Manzur and Fuentes, 1979). The widespread occurrence of pheromonal communication in lizards, the presence of precloacal pores in males with potential for deposition of pheromones on substrates, and observations that both sexes of *L. tenuis* tongue-flick the substrate after arriving at new locations (A. Labra, personal observation) suggest that *L. tenuis* might be responsive to conspecific chemical compounds. In this report, we present results of a study on intraspecific chemical recognition by *L. tenuis*. Because the rate of tongue-flick has been widely used as a good indicator of chemosensory investigation and discrimination of stimuli by squamates (Cooper and Burghardt, 1990a; Cooper, 1994, 1998), we recorded tongue-flicking performed by males and females in enclosures belonging to males or to females and within their own enclosures and control (unused) enclosures. During this investigation we addressed three questions: (1) Do individuals of *L. tenuis* show intraspecific

chemical recognition, particularly towards males, which have precloacal pores? (2) Does *L. tenuis* show seasonal changes in intraspecific chemical recognition? (3) Is chemical recognition dependent on sex?

METHODS AND MATERIALS

Animals and Their Maintenance. L. tenuis was collected at El Ingenio (30°46′S; 70°15′W; 1850 m) near Santiago, Chile. Twenty-one lizards (14 males, 7 females) were obtained during the postreproductive season in February and March 1997. During the breeding season, in October and November 1997, twenty-one lizards (11 males, 10 females) were collected. Animals were transported to the University of Chile and placed in a large outdoor vivarium exposed to ambient temperatures and natural photoperiod. Within the vivarium, lizards were housed individually in soil-filled plastic enclosures (30 × 19 × 10 cm) that were covered with thin plastic transparent mesh enabling lizards to climb. Because *L. tenuis* has been described as tree-dwelling, each enclosure contained a piece of water-washed tree bark (18 × 5 cm) of *Quillaja saponaria* (Rosaceae), one of the most common tree species at the capture site. The bark was used both as basking site and as shelter. Enclosures also contained a small bowl of water. Animals were fed mealworms every other day.

Experimental Design. Lizards remained in their enclosures for one week prior to experimentation. This allowed habituation to the experimental enclosures and deposition of chemical compounds, potentially active as pheromones. After the period, lizards were removed from their enclosures and maintained individually in fabric bags for 45 min; thereafter, their behaviors were recorded in two consecutive stages. In stage I, lizards were placed individually in one of the four experimental enclosure types: the empty home enclosure of a male (M), the empty home enclosure of a female (F), the home enclosure of the tested individual (H), or an empty control untreated enclosure (C). Thus, a lizard tested in a conspecific enclosure was exposed only to chemical signals and not to visual stimuli from a conspecific, since the owner was removed. After a lizard's behavior was recorded in stage I, it was removed and returned to its own fabric bag, where it remained for another 45 min. Thereafter, it was placed again in its home enclosure, and its behavior was recorded again (stage II). In some trials, while a lizard was maintained in its fabric bag between stages, its home enclosure was used to test the response of another individual in a conspecific enclosure. The process did not take longer than 20 min. Preliminary results indicated that the owner did not show differences in behavior upon returning to its home enclosure previously used by a conspecific.

The experimental enclosure was placed in a sunny patch, behind a curtain that had a hole through which the observer could watch the lizards. After the

first tongue-flick, the behavior was recorded for 10 min. The following variables were registered:

1. Tongue-flick (TF): the lizard extrudes and rapidly retracts its tongue, regardless of whether the tongue touches the substrate or is waved in the air.

2. Motion time: the time that animals remained in motion (minutes), which included adjustment in body posture, head movements (scanning), and displacements of the body's center of gravity through the habitat.

3. Latency to the first tongue-flick: the time period between placement of the lizard in the enclosure and onset of the first tongue-flick (minutes).

If the lizard retreated under the bark or failed to extrude the tongue within 45 min, the trial was canceled and postponed to a later date. Two males in the postreproductive season failed to tongue-flick and were discarded from the analysis. Only one two-stage trial per day was conducted for a given individual. Lizards had at least one day to rest before they were tested under a different experimental condition. Each animal was tested randomly in the four experimental conditions of stage I. With this design, the behavior of each animal was recorded in eight conditions (four in each stage). During experiments, handling was minimized to reduce stress and was similar in all trials. Experiments were restricted to late morning and early afternoon, at which times ambient temperatures were in the 30s (°C). When lowland *Liolaemus* have no constraints for thermoregulation, as was the case of *L. tenuis* in these experiments, lizards do not show seasonal changes in selected body temperature (Labra, 1998). Thus, experimental conditions ensured that lizards were fully active, not heat-stressed, and with similar body temperatures among seasons. All the animals were returned to the field after the experiments were finished.

In these experiments different enclosures were used instead of cotton swabs bearing conspecific chemicals (Cooper, 1998) because in pilot experiments with swabs lizards showed signs of stress and failed to show TF towards the swabs during more than 20 min.

Analysis. To test the effect of season, sex, condition (a total of eight conditions, four in each stage), and their interactions on number of tongue-flicks, motion time, and latency to the first tongue-flick, data were analyzed by parametric analysis of variance with a three-factor design and repeated measurements for condition (Edwards, 1968). After the ANOVA, Tukey's tests were used for individual comparisons. Even though two of the data sets were not normally distributed (female's latency in her own enclosure during breeding season and male's latency in the control during postreproductive season), and data of tongue-flick in males' enclosures showed heterogeneity of variance, ANOVA was used because it is robust enough to perform well even if the data deviate from the requirements of normality, homoscedasticity, and additivity (Zar, 1984). As a check of this procedure, a nonparametric approach was used. Data were ranked and ANOVA was used as described above with a posteriori test for multiple

comparisons (Zar, 1984). This latter approach was used to analyze differences among conditions, considering season and sex as a block and excluding variability within the block. Additionally, the original data were log transformed, and an ANOVA was performed. Since the results obtained with different approachs were consistent, only results of parametric tests were presented. Finally, Pearson correlations were used to test relationships between variables.

RESULTS

When animals were placed in any of the experimental enclosures, they spent a short time (about 1 min) breathing rapidly, possibly due to the stress of being handled. However, exploratory behavior began soon thereafter. There was a tendency towards longer latency to first tongue-flick by lizards placed in the control enclosures and shorter latency when they were tested in their home enclosures in stage I (Figure 1). Nevertheless, there was no significant effect of any factor or interaction of factors on latency to the first tongue-flick ($P > 0.05$).

Results for tongue-flick are presented in Table 1. During the breeding sea-

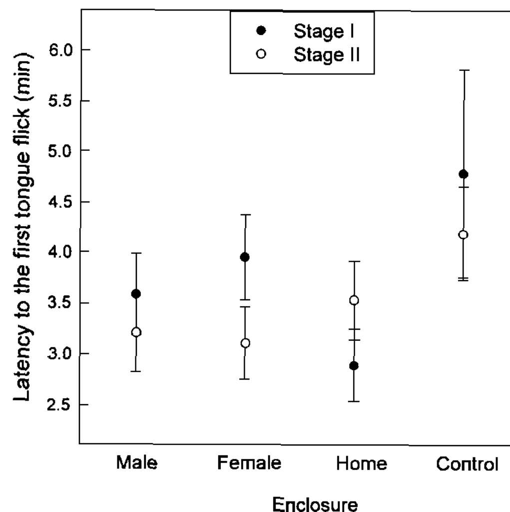

FIG. 1. Mean ± SE latency time to the first tongue-flick exhibited by *Liolaemus tenuis* in the two stages of the experiments. In stage I, animals were placed in one of the following conditions: male, female, home, or control enclosure. In stage II, animals were returned to their home enclosures after being tested in stage I. Test period lasted 10 min. Since sex and season did not have any effect in the latency to the first TF, data for both sexes and seasons were pooled.

TABLE 1. TONGUE-FLICKS MADE IN TWO SEASONS BY MALE AND FEMALE *Liolaemus tenuis* IN DIFFERENT CONDITIONS[a]

		Stage I				Stage II			
Season and sex	*N*	Male (M)	Female (F)	Home (H)	Control (C)	M-H	F-H	H-H	C-H
Post-reproductive									
Male	12	20.58 ± 3.57	19.08 ± 2.49	9.50 ± 0.81	21.17 ± 3.99	4.67 ± 1.22	5.33 ± 0.80	4.83 ± 1.21	5.58 ± 1.11
Female	7	15.86 ± 2.54	14.14 ± 2.84	9.14 ± 1.16	13.71 ± 2.35	7.71 ± 1.29	7.71 ± 1.82	6.43 ± 1.82	5.29 ± 1.11
Breeding									
Male	10	23.10 ± 2.15	32.40 ± 7.64	10.70 ± 1.40	25.40 ± 3.37	6.50 ± 0.99	6.50 ± 1.65	5.50 ± 1.08	6.30 ± 1.72
Female	11	22.09 ± 0.83	27.00 ± 2.46	13.45 ± 2.06	22.73 ± 2.18	5.45 ± 0.73	8.82 ± 1.17	6.45 ± 1.04	7.36 ± 0.54
Overall mean ± SE	40	20.80 ± 1.31	23.72 ± 2.38	10.82 ± 0.76	21.35 ± 1.69	5.88 ± 0.54	7.00 ± 0.67	5.73 ± 0.61	6.20 ± 0.59

[a]Stage I: Tested enclosures of Male (M), Female (F), Control (C) and the Home (H). Stage II: M-H: home enclosure after male's enclosure. F-H: home enclosure after female's enclosure, H-H: home enclosure after home enclosure and C-H: home enclosure after control enclosure. N = sample size. The period of recording was 10 min. Values are means ± standard errors. The overall mean of each treatment is indicated also.

TABLE 2. REPEATED-MEASURES ANALYSIS OF VARIANCE OF EFFECTS OF SEX (MALE VERSUS FEMALE), SEASON (BREEDING VERSUS POSTREPRODUCTIVE), EXPERIMENTAL CONDITION (DIFFERENT ENCLOSURES, INCLUDING RESULTS OF STAGES I AND II), AND THEIR INTERACTION UPON TONGUE-FLICK

Source of variation	*F*	*df*	*P*
Between subjects			
Season	6.833	1,36	0.013
Sex	0.373	1,36	0.545
Season × sex	0.117	1,36	0.735
Within subjects			
Condition	51.626	7,252	≪0.0001
Condition × season	4.366	7,252	0.0001
Condition × sex	2.060	7,252	0.048
Condition × season × sex	0.465	7,252	0.859

son lizards exhibited a significantly higher number of TF than during the postreproductive season (Table 2). Males and females did not show a significant difference in TF (Table 2). The experimental condition had a significant effect upon tongue-flicking. A Tukey test indicated that lizards exhibited significantly more TF in any novel environment than in their home enclosure (compare in Table 1 data for H with data for M, F, or C), and their were no significant differences of TF among M, F, and C enclosures. The number of TF when lizards were placed for the first time in their home enclosure was significantly higher than the number of TF recorded in stage II (compare in Table 1 data for H with M-H, F-H, H-H, and C-H). TF recorded in the different conditions of stage II did not differ significantly among them, and they were significantly lower than those recorded in any stage I condition (Table 1).

The interaction between season and experimental condition was significant (Table 2); female enclosures elicited significantly more TF in the breeding than in the postreproductive season, and in the latter season, lizards exhibited a similar number of TF in any novel enclosure (Figure 2). The interaction between sex and condition was significant (Table 2). Males showed more TF in a novel enclosure than females, but not in home enclosures in either stage. However, the interaction between season, sex, and experimental condition was not significant (Table 2).

The number of TF performed in the air during both seasons was much lower than those performed on the substrate, 25 (1.5% of total) and 4 (0.16% of total) during postreproductive and breeding season, respectively. A Pearson correlation of the pooled data of the number of TF and latency to the first tongue-flick indicated that the two variables were not correlated ($r = 0.063$; $P = 0.26$; $N = 320$).

The first TF was always preceded by movements of the head (scanning).

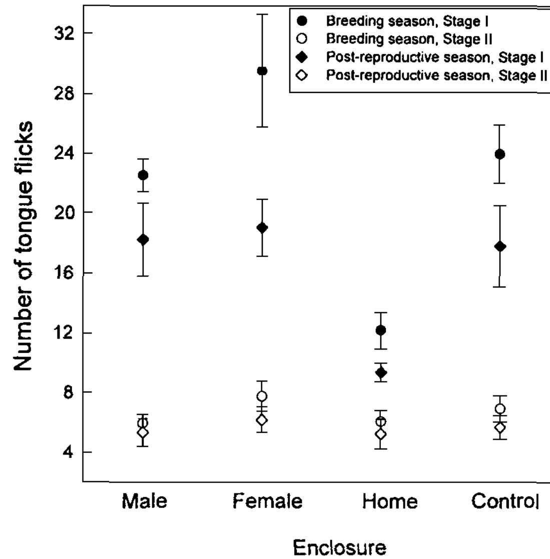

FIG. 2. Mean ± SE number of tongue-flicks exhibited by *Liolaemus tenuis* in two seasons (postreproductive and breeding) in the two stages of the experiments. The stages are explained in Figure 1 legend. Test period lasted 10 min. Since males and females did not show differences in TF, data for both sexes were pooled.

However, the only factor affecting motion time was experimental condition (Table 3). Animals spent significantly more time performing some movement during stage I than during stage II (Figure 3). Nevertheless, the time that animals spent moving in their home enclosures in stage I did not differ from any other experimental condition (Figure 3).

Motion time and TF showed a significant and positive relationship ($r = 0.58$; $P < 0.05$; $N = 320$). In contrast, motion time was not correlated with the latency to the first tongue-flicking ($r = 0.091$; $P = 0.105$; $N = 320$).

DISCUSSION

The experiments described show that both sexes of *L. tenuis* respond differently to their home enclosures than to enclosures belonging to conspecifics during both seasons. Both sexes also showed no overall differences in the behaviors recorded. Lizards showed lower TF in their home enclosures than in any novel enclosure. The close relationship reported between tongue-flicking and vomerolfaction (Cooper, 1998) suggests that chemical compounds or vomodors (*sensu* Cooper and Burghardt, 1990b) are likely involved in this home recog-

TABLE 3. REPEATED-MEASURES ANALYSIS OF VARIANCE OF EFFECTS OF SEX (MALE VERSUS FEMALE), SEASON (BREEDING VERSUS POSTREPRODUCTIVE), EXPERIMENTAL CONDITION (DIFFERENT ENCLOSURES, INCLUDING RESULTS OF STAGES I AND II), AND THEIR INTERACTION UPON MOTION TIME

Source of variation	*F*	*df*	*P*
Between subjects			
Season	1.121	1,36	0.297
Sex	3.231	1,36	0.081
Season × sex	0.001	1,36	0.982
Within subjects			
Condition	8.493	7,252	≪0.001
Condition × season	0.894	7,252	0.512
Condition × sex	0.786	7,252	0.599
Condition × season × sex	0.755	7,252	0.626

nition, probably recognition of self-produced pheromones or pheromones produced by other individuals. Pheromonally mediated self-recognition might allow the maintenance of a territory at a lower cost by reducing visual displays and/or aggression among conspecifics. These factors may be important selective forces

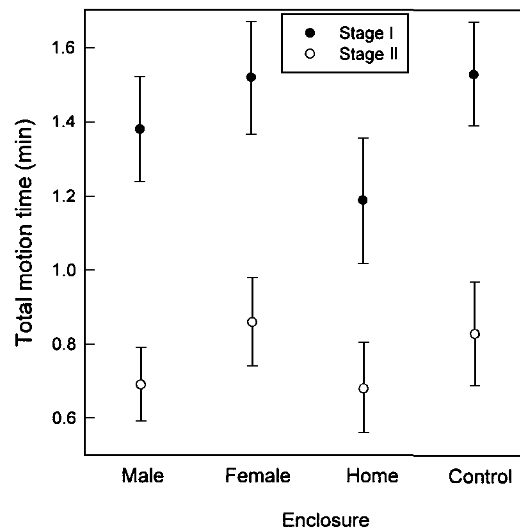

FIG. 3. Mean ± SE total motion time exhibited by *Liolaemus tenuis* in the two stages of the experiments. The stages are explained in Figure 1 legend. Test period lasted 10 min. Since sex and season did not have any effect in the latency to the first TF, data for both sexes and seasons were pooled.

for chemical self-recognition, which has been observed in different lizard families such as Iguanidae (Alberts, 1992), Phrynosomatidae (De Fazio et al., 1977), Polychrotidae (Greenberg, 1985), Scincidae (Graves and Halpern, 1991), and now in Tropiduridae.

The lower motion time coupled with reduced TF in stage II indicates an overall reduction of exploratory behavior. This might be due to habituation to handling (Gómez et al., 1993), or be a consequence of recent chemosensory investigation during stage I. During the habituation period lizards only explored their territories, having no possibility to contrast signals of their territories with those of other lizards. Animals placed in any enclosure in stage I might "establish" differences or similarity with signals of their home enclosures based on the recall and contrast of chemicals or/and other signals.

Seasonal differences in chemical exploratory behavior were observed that were not coupled with differences in motion time. During the postreproductive season, the overall rate of TF was lower than in the breeding season and lizards did not discriminate among the different unfamiliar enclosures (Figure 2). In contrast, in reproductive season both sexes showed recognition toward females, and female enclosures elicited more TF. These seasonal behavioral differences might be related to seasonal physiological differences. In the autumn (postreproductive season), *L. tenuis* is preparing itself for hibernation and probably conspecific recognition is not a priority. In contrast, a higher chemical exploratory behavior exhibited during the breeding season would be important for reproduction-related behaviors, such as mate searching and territory establishment (Moore and Lindlezy, 1992; Whittier and Tokarz, 1992). For a reproductive and polygynous *L. tenuis* male, the detection of a female would be important, since males may initiate behaviors to attract a female to its territory. Consexual chemical recognition of females can be understood considering that females exhibit agonistic behavior inside a male's territory and tend to establish hierarchies (Manzur and Fuentes, 1979). Chemical recognition might help to reduce agonistic encounters among females inside a male's territory.

Intraspecific recognition in *L. tenuis* appears to be mainly mediated by nonvolatile substances, since most TF were performed to the substrate rather than to the air. Tongue flicking is a requisite for gathering and transporting these substances to the vomeronasal organ (Halpern, 1992; Cooper, 1994). Additionally, the latency to the first TF was similar among experimental conditions, including the control. If the chemical cues had been volatile, a relationship between latency to the first TF and the number of TF (Cooper et al., 1994) or differences in TF between the conspecific and control enclosures would be expected. Alternatively, it is known that lizards can also recognize a territory by visual cues, such as feces (Duvall et al., 1987) or UV light absorbed by pheromones (Alberts, 1989). Since *L. tenuis* had a similar number of TF in conspecific enclosures and enclosures lacking visual signals of the presence of other lizards, visual cues probably were

not an important source of information in these experiments. For the recognition of the home enclosure, minimal structural or chemical differences between the bark present could have been used by lizards as signals to determine a site as familiar. However, bark was not the main site used for chemical recognition, since most of TF were performed directly to the soil and less than 30% to the bark.

It remains unclear where the pheromones involved in intraspecific chemical recognition in *L. tenuis* are produced. Tropidurid lizards lack femoral glands (Frost and Etheridge, 1989), one of the most studied pheromone-producing glands in lizards (Mason, 1992; Alberts, 1993). However, the precloacal pores of *L. tenuis* males have a histology similar to femoral glands (Labra and Niemeyer, unpublished data), and they are a potential source of pheromones for males. Because neither sex displayed higher TF in male enclosures, even in the breeding season, it is not clear whether precloacal secretions of *L. tenuis* males are involved in territorial marking, male recognition, and female attraction. It is possible that pheromonal products might be detected and identified without prolonged chemosensory investigation.

Females of *L. tenuis* lack precloacal pores and urodaeal glands are a hypothetical source of pheromones for females. These glands, present only in the cloaca of females (Mason, 1992), produce lipid secretions acting as pheromones in *Eumeces laticeps* (Cooper et al., 1986) and *Gerrhosaurus nigrolineatus* (Cooper and Garstka, 1987). Further research is needed to determine the site of pheromone production in both sexes of *L. tenuis.*

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OLFACTORY RECEPTION OF CONSPECIFIC AGGREGATION PHEROMONE AND PLANT ODORS BY NYMPHS OF THE PREDATOR, *Podisus maculiventris*

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Abstract—Olfactory reception of 23 odorants, including plant volatiles and male-produced aggregation pheromone, by third and fifth instars of the spined soldier bug (SSB) *Podisus maculiventris* was investigated by using electroantennograms (EAGs). Both nymphal stages were sensitive to male-produced aggregation pheromone components (*E*)-2-hexenal, benzyl alcohol, and α-terpineol. The plant volatile, (*E*)-2-hexen-1-ol (a chemical known to be released by plants in response to prey feeding over the short-term), elicited the largest EAGs of all volatiles tested. While third instars were sensitive to nonanal, only fifth instars responded to both nonanal and (±)-linalool, both compounds released systemically by plants in response to feeding by potential prey. Antennal extirpation experiments showed that sensilla responsive to hexan-1-ol, (*E*)-2-hexenal, and α-terpineol are situated mainly on the terminal antennal segment. The results support the hypothesis that *P. maculiventris* nymphs use both plant volatiles and pheromone components in locating potential prey and other behaviors.

Key Words—Plant volatiles, pheromone, odor perception, nymphs, predators, spined soldier bug, *Podisus maculiventris*.

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INTRODUCTION

The spined soldier bug (SSB), *Podisus maculiventris* (Say), is an important predator throughout North America. It has a wide prey range, including several important vegetable pests such as the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae) (McPherson, 1982; Hough-Goldstein and Keil, 1991; Thomas, 1992; Hough-Goldstein and McPherson, 1996). SSB males secrete an aggregation pheromone from a pair of large dorsal abdominal glands (DAGs) opening between abdominal tergites 3 and 4. The pheromone is a blend of seven compounds: principally (*E*)-2-hexenal, α-terpineol, and benzyl alcohol with lesser amounts of (±)-linalool, terpinen-4-ol, *trans*- and *cis*-piperitol (Aldrich et al., 1984b; Aldrich, 1988). Differing from adults, SSB nymphs have three pairs of DAGs opening between abdominal tergites 3 and 4, 4 and 5, and 5 and 6. While (*E*)-2-hexenal is the predominant component in the 3–4 DAGs, DAGs 4–5 and 5-6 contain *n*-tridecane, (±)-linalool, and (*E*)-4-keto-2-hexenal (Aldrich et al., 1984b). Similar compounds occur in extracts of exuviae of fifth-instar *P. nigrispinus* and *P. distinctus* (Aldrich et al., 1997). While the function of these compounds is not well known, they might be used by immature insects as cues for locating food (Aldrich, 1995) and conspecifics, or by parasitoids as kairomones for locating potential hosts (Aldrich et al., 1984a,b; Aldrich, 1995). In laboratory and field experiments, SSB nymphs are attracted to synthetic pheromone of conspecific males (Sant'Ana et al., 1997).

In addition to pheromone compounds, odors emitted by plants may be important cues for location of prey or hosts by other insect predators or parasitoids (Vet and Groenewold, 1990; Dicke et al., 1990). Stimuli derived from plants provide cues for long-range attraction, but as predictors of prey location they are less reliable than volatiles emitted by prey (Vet and Dicke, 1992). However, for generalist predators, prey odors may be less important than volatiles emitted from plants, which, especially during herbivore attack, act as fundamental cues attracting natural enemies (Turlings et al., 1991; McCall et al., 1993; Mattiacci et al., 1994; Vaughn et al., 1996; Dickens, 1999).

Olfaction has been investigated in several adult Hemiptera including *Oncopeltus fasciatus* (Lygaeidae) (Pantle and Feir, 1976), *Lygus lineolaris* (Miridae) (Chinta et al., 1994; Dickens et al., 1995, 1998; Dickens and Callahan, 1996), *Podisus maculiventris* and *P. nigrispinus* (Sant'Ana and Dickens, 1998). However, in only few studies were electrophysiological responses recorded from immature stages (Levinson et al., 1974; Dickens et al., 1995), and no study has investigated responses of immature stages in detail. Successful use of semiochemicals to manipulate predator–prey interactions for biorational pest control depends on knowledge of the compounds that mediate intra- and interspecific interactions and their specific behavioral roles.

We report here electroantennogram (EAG) investigations of chemoreceptive

responsiveness and sensitivity of SSB nymphs to volatiles emanating from measured amounts of adult aggregation pheromone components and plant volatiles. Receptors for pheromone components and plant volatiles also were localized to specific antennal segments in extirpation experiments.

METHODS AND MATERIALS

Insects. SSBs were reared in a growth room at 26 ± 2°C, 60 ± 10% relative humidity, and 16L : 8D photoperiod, at USDA, ARS, Insect Chemical Ecology Laboratory, Beltsville, Maryland. Experimental insects were provided with water vials, green bean pods, and pupae of *Tenebrio molitor* (Coleoptera: Tenebrionidae).

Odorous Stimuli. Chemicals were chosen based on their presence in the 3–4 dorsal abdominal gland (DAG) of SSB and prey habitat (p-h) (Table 1). Chemical purity was determined by gas chromatography. Also tested were SSB gland extract (DAG), odor of *Heliothis virescens* larvae (a potential prey; ether extract of early fourth instar larvae ca. 60 larvae/ml), and a synthetic pheromone blend of SSB males, composed of: 53.6% (*E*)-2-hexenal, 40% α-terpineol, 4.9% benzyl alcohol, 0.9% (±)-linalool, and 0.5% terpinen-4-ol.

Stimulus dilutions in nanograde hexane were delivered from glass odor cartridges (80 mm long × 5 mm ID) as 5-μl aliquots on Whatman No. 1 filter paper measuring 7 mm × 18 mm. Odor cartridges were oriented towards the antenna from a distance of 1 cm. Odor molecules evaporating from the filter paper were carried over the preparation by dry, charcoal-filtered, hydrocarbon-free air. Stimulus duration was 1 sec. Airflow was 1 liter/min. Interstimulus time intervals of 2–3 min were allowed for recovery of the EAG. Air around the experimental set up was continuously exhausted.

Because of the wide range in volatilities of test compounds (see Kovats indices, Table 1) (Kovats, 1958), only relative comparisons can be made between the odorous stimuli, except for closely related compounds.

Electrophysiology. Electroantennogram techniques utilized in these studies were a modification of previous techniques and are described in detail elsewhere (Schneider, 1957; Payne, 1970, Sant'Ana and Dickens, 1998). Briefly, Ag–AgCl capillary electrodes filled with *Drosophila* ringer (NaCl 7.5 g/liter, KCl 0.35 g/liter, and $CaCl_2$ 0.21 g/liter) were used. Intact insects were immobilized on a cork block by using adhesive tape. After prepuncturing with a tungsten needle, the recording electrode was inserted into the distal antennal segment, and an indifferent electrode was placed into the proximal segment. The signal was amplified by a Grass P-16 microelectrode DC preamplifier and viewed on a Tektronix 5111A storage oscilloscope. EAGs were recorded on a stripchart recorder.

Experimental Protocol. Two sets of experiments were performed in order to elucidate the selectivity and sensitivity of the antennal receptors of nymphs *P.*

TABLE 1. CHEMICALS USED IN EAG EXPERIMENTS, THEIR PURITY, SOURCE, KOVATS RETENTION INDICES, AND BIOLOGICAL PRESENCE

Chemicals	Purity (%)	Source[a]	Kovats indices (I_k)[b]	Identified from insect (I) or prey habitat (P)[c]
Green-leaf volatiles				
(*E*)-2-Hexenal	>90	A	854	I 1/P 2, 3, 4, 5, 6, 7
Hexanal	>94	A	801	P 7
1-Hexanol	99	A	851	P 2, 7
(*E*)-2-Hexenol	>99	A	887	P 2, 3, 4, 5, 7
(*E*)-2-Hexenoic acid	100	A	na[d]	P 7
(*E*)-2-Hexenyl acetate	100	C	na	P 7
Monoterpenes				
Myrcene	>85	A	992	P 3, 4, 5, 6
(±)-Linalool	>99	A	1100	I 1/P 3, 4, 5
Nerol	>98	B	1251	P 2, 5
Geraniol	>88	B	1275	P 2, 3, 4, 5
α-Terpineol	>97	A	1195	I 1/P 2
Terpinen-4-ol	>94	A	1182	I 1
trans-Piperitol	>60	D	na	I 1
Benzenoid compounds				
Benzyl alcohol	100	A	1039	I 1/P 2
Benzaldehyde	>89	A	968	I 1/P 4
2-Methyl benzyl alcohol	>99	C	na	
Aliphatic aldehydes				
Heptanal	>90	A	903	P 3, 5, 7
Octanal	100	A	1006	P 7
Nonanal	>96	A	1104	P 3, 5
Tetradecanal	>60	C	1608	I 1 (nymphs)

[a]A: Aldrich Chemical Co., Milwaukee, Wisconsin; B: Pfaltz & Bauer, Inc. Stamford, Connecticut; C: Fluka Chemical AG, Buchs, Switzerland; D: PCR Research Chemical Inc., Gainesville, Florida.
[b]Kovats retention indices on DB-5 column (Acree and Arn, 1997). Value for tetradecanal interpolated from values for series of aliphatic aldehydes.
[c]1: Aldrich et al. (1984a); 2: Hedin et al. (1971); 3: Hedin et al. (1973); 4: Hedin et al. (1975); 5: Hedin et al. (1976); 6: Loughrin et al. (1994); 7: Van Straten and Maarse (1983).
[d]Not available.

maculiventris for male-produced pheromone components, prey–habitat volatiles, and a prey odor. A third experiment was performed to localize receptors for one plant odor and two pheromone components to specific segment(s). For the experiments, third and fourth instars were chosen. The third instar was chosen as it is the earliest stage that is released in the field to control pests; the fifth instar was selected since it is the stage just before adulthood and was shown to respond behaviorally to the male-produced attractant (Sant'Ana et al., 1997).

In the first series of experiments, the general responsiveness of antennal receptors to individual odorants was measured by recording EAGs to volatiles

emanating from 5-μg stimulus loads of each compound. Presentation of each odorant was randomly ordered for each sample. Six replicates were obtained for both third and fifth instars.

In the second series of experiments, odorants were selected for more detailed examination based on data obtained in the initial experiments. Dose–response curves were constructed from EAGs elicited by serial dilutions of each compound (0.005–500 μg/μl). Serial dilutions were presented in order from the lowest to the highest dose. Three replicates were obtained for fifth instars.

A third series of experiments was performed in order to localize pheromone and plant odor receptors on the antennae of fifth instars. EAGs were recorded from nymphs in which the terminal segment of the four-segmented antenna was clipped and nymphs in which both the terminal and penultimate segments were clipped. EAGs obtained in this experiment were compared to EAGs obtained from intact antennae. The recording electrode was always placed at the end of the last intact antennal segment. All measurements were performed in triplicate with 50-μg stimulus loads of hexan-1-ol (a common plant odor), and (*E*)-2-hexenal and α-terpineol (pheromone components). Different individuals were used for each treatment.

Hexan-1-ol (50-μg stimulus load) was used as a standard to normalize all responses, so that responses within an individual and among individuals could be compared (Payne, 1975). Stimulation with the standard preceded and followed every two experimental stimuli. Millivolt responses were converted into a percentage of the mean of the two nearest responses to the standard (Dickens, 1978). Control stimulation, which used filter paper impregnated with 5 μl of the hexane solvent, was made at the beginning and at the end of each preparation. The mean response to the control was subtracted from each EAG. Maximal depolarization of the EAG during the stimulation period was used as a measure of antennal stimulation.

The threshold was considered to be the lowest dose at which the lower limit of the standard error of the mean response was greater than the upper limit of the standard error for the lowest dilution tested. Saturation level was taken as the highest dose at which the mean response was equal to or less than the succeeding dose.

Statistical Analyses. EAGs were compared statistically by using analysis of variance and Duncan's multiple range test (Duncan, 1955). Differences between points on dose–response curves were compared for significant differences in the fifth instar by using the *t* test for two means (Ostle, 1969).

RESULTS AND DISCUSSION

The mean electroantennogram response to the hexan-1-ol standard (50-μg stimulus load) in SSB third-instar antennae [0.15 mV ± 0.004 (SE); $N = 22$) was smaller than that obtained for fifth-instar antennae (0.26 ± 0.003 mV; $N = 22$)

TABLE 2. MEAN EAGS OF SIX THIRD INSTARS AND SIX FIFTH INSTARS OF *Podisus maculiventris* TO 50-μg STIMULUS LOAD OF COMPONENTS OF ADULT AGGREGATION PHEROMONE, CONSPECIFIC CHEMICALS, AND PLANT VOLATILES[a]

Third instars		Fifth instars	
Chemical	Mean EAGs[b]	Chemical	Mean EAGs[b]
(*E*)-2-Hexen-1-ol	277.2a	(*E*)-2-Hexen-1-ol	287.5a
(*E*)-2-Hexenal	172.5b	Nonanal	123.6b
Synthetic pheromone blend	171.5b	Heptanal	108.9bc
(+)-α-Terpineol	147.2b	**(*E*)-2-Hexenal**	108.8bc
Nonanal	145.3b	(+)-α-Terpineol	92.5bcd
Benzaldehyde	140.6b	**Synthetic pheromone blend**	79.1cde
Heptanal	139.3b	Dorsal abdominal gland	60.6def
Benzyl alcohol	127.8bc	**(+)-Linalool**	57.5def
Hexanal	105.7bcd	**Benzyl alcohol**	49.3ef
Terpinen-4-ol	65.9cde	Terpinen-4-ol	48.6fe
Dorsal abdominal gland	53.9ed	*trans/cis*-Piperitol	48.5ef
trans/cis-Piperitol	48.1ed	(*E*)-2-Hexenoic acid	47.9ef
2-Methyl benzyl alcohol	25.1e	Heptanal	46.9ef
Tetradecanal	22.9e	**Benzaldehyde**	44.6ef
Nerol	13.8e	2-Methyl benzyl alcohol	43.9efg
Myrcene	12.4e	Nerol	40.7efg
(*E*)-2-Hexenoic acid	0e	(*E*)-2-Hexenyl acetate	39.7efg
(*E*)-2-Hexenyl acetate	0e	Octanal	37.2efg
(+)-Linalool	0e	Myrcene	31.9fg
Octanal	0e	Tetradecanal	27.5fg
Geraniol	0e	Geraniol	15.2fg
Heliothis virescens larval extract	0e	*Heliothis virescens* larval extract	0g

[a]Numbers followed by different letters are significantly different within sex ($P < 0.05$; Duncan's multiple range test). Response to chemicals in bold type differ significantly between instars.
[b]EAGs are represented as a percent response to the standard.

($P < 0.001$). Since the amplitude of EAGs is thought to be related to the relative number of receptor cells responding to an odor stimulus (Payne, 1975), smaller EAGs observed for third instars suggest a smaller number of antennal olfactory sensilla and resident receptor cells.

Selectivity. EAGs in response to individual chemicals were different between third- and fifth-instar nymphs ($P < 0.05$) (Table 2). (*E*)-2-Hexen-1-ol was the most effective stimulus tested for both instars. This compound is a potent EAG stimulus for several other species belonging to the orders Lepidoptera (Dickens et al., 1993), Coleoptera (Visser, 1979; Dickens, 1989; Dickens and Boldt, 1984), Hymenoptera (Light et al., 1992), Diptera (Light et al., 1988), and other Hemiptera including adults of *L. lineolaris* (Chinta et al., 1994), *P.*

maculiventris, and *P. nigrispinus* (Sant'Ana and Dickens, 1998). Both instars were also responsive to (*E*)-2-hexenal, the predominant odor of the male-produced aggregation pheromone in SSB. Both (*E*)-2-hexen-1-ol and (*E*)-2-hexenal are common volatiles present in the odor of green leaves of plants (Hatanaka, 1993; Croft et al., 1993; Loughrin et al., 1994) and may be released by the plant over the short term in response to insect feeding (Loughrin et al., 1995; Bolter et al., 1997).

(*E*)-2-Hexenal may be a bifunctional pheromone for SSB nymphs. In another hemipteran, the cabbage bug, *Eurydema rugosum* (Pentatomidae), laboratory experiments showed that large quantities of (*E*)-2-hexenal release alarm behavior, while gradual release of small amounts of the chemical elicits aggregation (Ishiwatari, 1974, 1976). Levinson et al. (1974) reported that escape in adults and nymphs of *Cimex lectularius* (Hemiptera: Cemicidae) is induced by (*E*)-2-hexenal and depends on its concentration in the air. Thus, (*E*)-2-hexenal may induce alarm behavior in SSB nymphs. Alternatively, Aldrich et al. (1984b) suggest that (*E*)-2-hexenal may act as a short-range pheromone that aggregates young SSB nymphs, and other compounds such as (±)-linalool would be responsible for long range aggregation, possibly used by older nymphs to recongregate in the periods of molting.

Although third instars were unresponsive to (±)-linalool, geraniol, octanal, (*E*)-2-hexenoic acid, (*E*)-2-hexenyl acetate, and *Heliothis virescens* larval extract, significant EAGs were observed in the fifth-stage nymphs to the same volatiles, except the *H. virescens* extract (Table 2). This suggests that (±)-linalool and other compounds would not be involved in chemical communication in earlier SSB instars, but may be important as chemical signals in subsequent nymphal stages. Thus, each nymphal stage may use different olfactory cues and possess different types of receptor cells.

Among monoterpenes, α-terpineol elicited the largest EAGs (Table 2). Field experiments revealed the blend of (*E*)-2-hexenal, α-terpineol, and benzyl alcohol as a long-range attractant for male and female SSB (Aldrich, 1988). Subsequent studies in the wind tunnel and field revealed that not only adults, but also conspecific nymphs, are attracted by this blend (Sant'Ana et al., 1997). Leal et al. (1995) noted the attractiveness of *Riptortus clavatus* (Hemiptera, Heteroptera: Alydidae) nymphs to male-released pheromone and suggested that the pheromone signal would enable immature stages to locate food sources at the lowest energy cost. Pheromone emitted by SSB males may be used by immature SSB in a similar way, since the presence of pheromone from conspecific males would be associated by nymphs with available prey (Aldrich, 1995). Alternatively, plants damaged by potential prey, e.g., the Colorado potato beetle (*Leptinotarsa decemlineata*), release (*E*)-2-hexenal (Schütz et al., 1997) which would facilitate prey location but in a different context.

Chemicals emanating from plants often enhance insect responses to

pheromones (Dickens, 1989; Dickens et al., 1990; Landolt and Phillips, 1997). In *Podisus*, components of the pheromone blend are also common plant volatiles, so it is reasonable to conclude that two sources of volatiles interact in insect strategies to locate food, mates, or oviposition sites. We can infer that the *Podisus* species studied might not have specific receptors that encode plant or pheromonal information. Clearly, behavior elicited by these volatiles must be due to the concentration of the specific volatile and the context in which it is encountered, e.g., presence with other odorants or temporal variation.

In both adult SSB (Sant'Ana and Dickens, 1998) and fifth instars, EAGs elicited by octanal are significantly smaller than EAGs elicited by heptanal and nonanal (Table 2). Responsiveness of antennal receptors of insects to saturated aldehydes increases as the chain length increases until a peak is reached at six or seven carbons, after that responsiveness declines due to decreasing volatilities with increasing of the chain length (Dickens, 1984; Dickens and Boldt, 1984; Light et al., 1988; Chinta et al., 1994). Enhanced responsiveness to nonanal may represent specific receptors in the nymphs for this compound or a closely related compound. Since nonanal is released systemically from the foliage of potato plants in response to feeding by the Colorado potato beetle (Bolter et al., 1997; Schütz et al., 1997), it could signal the presence of potential prey.

Of the two blends of pheromone tested, the synthetic blend elicited larger EAGs in third instars than did extract of male DAGs, while significant differences to these blends were not observed in fifth instars. Differential responses to the synthetic pheromone blend compared to the pheromone extract by third instars indicates additional components in the DAG extract that are detected by fifth instars but not by third instars.

Sensitivity and Responsiveness. In general, the amplitude of EAGs of fifth-instar SSB to serial stimulus loads of principal components of the adult sex pheromone and plant odors increased with increasing stimulus loads (Figure 1). Antennae of fifth instars were most sensitive to nonanal and (±)-linalool, reaching the threshold at a 0.5-μg stimulus load (Figure 1C and F). Since the low threshold might indicate the ability of the insect to perceive the odor at greater distances from its source (Dickens, 1984), fifth-instar nymphs would detect these volatiles at lower concentrations than the other chemicals. Our results support the hypothesis that (±)-linalool, which is a pheromone component, would be used by older nymphal stages as a long-range attractant to recongregate prior to molting for protection (Aldrich et al., 1984b). Alternatively, both linalool and nonanal are released systemically from plants in response to insect feeding (Bolter et al., 1997; Schütz et al., 1997) and, thus, may cue these predators preveniently to a meal.

Nymphs were less sensitive to α-terpineol and benzyl alcohol than either nonanal or (±)-linalool; threshold responses to these compounds were 5 μg (Figure 1D and E). The active space of these pheromone components may be less

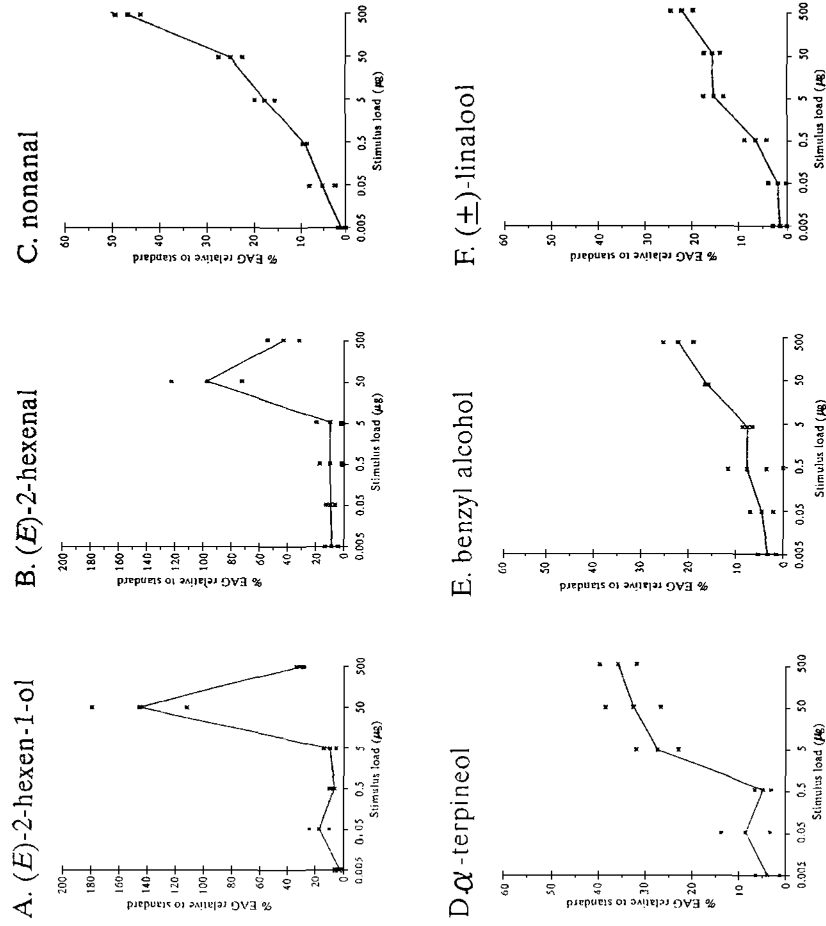

FIG. 1. Dose–response curves constructed from mean EAGs of fifth-instar *Podisus maculiventris* to serial stimulus loads of selected insect and plant odorants: (A) (*E*)-2-hexen-1-ol, (B) (*E*)-2-hexenal, and (C) nonanal, (D) α-terpineol, (E) benzyl alcohol, and (F) (±)-linalool. Markers above and below points on dose–response curves represent ±SE.

TABLE 3. MEAN EAGS ($N = 3$)[a]

Chemical	EAG (Mean ± SE, (mV))[b]		
	Whole antenna	Segments 1–3	Segments 1–2
Hexan-1-ol	0.41 ± 0.06a	0.1 ± 0.00b	0c
(+)-α-Terpineol	0.26 ± 0.03a	0b	0b
(*E*)-2-Hexenal	0.66 ± 0.14a	0.04 ± 0.02b	0c

[a]EAGs were recorded from whole antennae (four segments) of fifth-instar *Podisus maculiventris* antennae with the terminal segment removed (segments 1–3), and antennae with both the terminal and penultimate segments removed (segments 1–2) in response to the plant odor, hexan-1-ol, and two pheromone components, α-terpineol and (*E*)-2-hexenal (50-μg stimulus load).
[b]Numbers followed by different letters are significantly different within sex ($P < 0.05$; Duncan's multiple range test).

than for (±)-linalool. Antennal receptors in nymphs were least sensitive to (*E*)-2-hexen-1-ol and the corresponding aldehyde (threshold, 50-μg stimulus load) (Figure 1A and B). Acceptor saturation in antennae of SSB fifth instars was reached at a 500-μg stimulus load for both (*E*)-2-hexen-1-ol and (*E*)-2-hexenal. The same threshold responses and saturation levels to these compounds were also found in SSB and *P. nigrispinus* adults (Sant'Ana and Dickens, 1998); however, it was not observed in other species such as *Anthonomus grandis* (Coleoptera: Curculionidae (Dickens, 1989) and *Lygus lineolaris* (Hemiptera: Miridae) (Chinta et al., 1994). The strong response of fifth instars to the 50-μg stimulus load and, subsequently, saturation at 500 μg suggests that these aliphatic alcohols and aldehydes may be important as short-range signals for SSB.

Localization of Responsive Olfactory Sensilla. Maximal responses to the three components tested, hexan-1-ol, (*E*)-2-hexenal, and α-terpineol, were recorded from whole antennae of fifth instars (Table 3). EAGs to hexan-1-ol and (*E*)-2-hexen-1-ol decreased dramatically when the terminal segment was extirpated (i.e., segments 1–3); response to α-terpineol was eliminated under these conditions. Removal of both distal and penultimate segments of fifth instars decimated response to all compounds. Thus, receptor cells responsible for detection of (*E*)-2-hexenal, hexan-1-ol, and α-terpineol in fifth instars are situated mainly on the terminal antennal segment. This correlates with the fact that the antennae in earlier instars of SSB have only a few sensilla located mainly on the terminal segment, while later instars possess increased numbers of sensilla on the three distal segments but mainly on the terminal segment (Sinitsina and Krutov, 1996; Dickens and Sant'Ana, unpublished). Sensilla in adults for detection of the same compounds as tested in the nymphs are distributed over the distal three segments of the five-segmented antenna (Sant'Ana and Dickens, 1998). Thus, while electrophysiological responses to these odorants decrease as individual segments are removed, significant EAGs could still be recorded after removal of the two distal segments.

In conclusion, third and fifth instars of SSB detect components of the aggregation pheromone produced by adult males. These immature stages are also sensitive to a number of plant volatiles that are released by the plant in response to insect feeding both over the short-term and systemically over time (Bolter et al., 1997; Schütz et al., 1997). Both pheromone components and plant volatiles may be used as chemical signals to orient immatures to potential prey and/or to facilitate aggregations to afford protection of vulnerable nymphs undergoing molting. Knowledge of the mechanisms by which SSB nymphs detect potential chemical signals and the identity of them may be useful in shepherding both immatures and adults as components of integrated pest management as shown for the Colorado potato beetle (Sant'Ana et al., 1997).

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PLANT LATEX AND FIRST-INSTAR MONARCH LARVAL GROWTH AND SURVIVAL ON THREE NORTH AMERICAN MILKWEED SPECIES

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Abstract—First-instar larvae of the monarch butterfly, *Danaus plexippus*, a milkweed specialist, generally grew faster and survived better on leaves when latex flow was reduced by partial severance of the leaf petiole. The outcome depended on milkweed species and was related to the amount of latex produced. The outcome also may be related to the amount of cardenolide produced by the plants as a potential chemical defense against herbivory. Growth was more rapid, but survival was similar on partially severed compared with intact leaves of the high-latex/low-cardenolide milkweed, *Asclepias syriaca*, whereas both growth and survival were unaffected on the low-latex/low-cardenolide milkweed *A. incarnata*. On the low-latex/low-cardenolide milkweed *A. tuberosa*, both growth and survival of larvae were only marginally affected. These results contrast sharply to previous results with the milkweed, *A. humistrata*, in Florida, which has both high latex and high cardenolide. Larval growth and survival on *A. humistrata* were both increased by partially severing leaf petioles. Larval growth rates among all four milkweed species on leaves with partially severed petioles were identical, suggesting that latex and possibly the included cardenolides are important in first-instar monarch larval growth, development, and survivorship.

Key Words—*Asclepias*, cardenolide, *Danaus plexippus*, growth rate, latex, laticifer, milkweed, neonate larvae, plant defense, survival.

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INTRODUCTION

Small, toxic molecules in plants have been argued to be effective defenses against generalist herbivores but not against specialist herbivores (Feeny, 1976; Rhoades and Cates, 1976). Both apparency theory and optimal defense theory argue that toxic products of secondary plant metabolism, such as cyanogenic glycosides, alkaloids, and cardenolides, are effective defenses against most generalist herbivores. The generalization implicit in these theories is that "plant toxins" have little effect on specialists, and this has since been corroborated by evidence that "plant toxins" can have little negative impact on the growth and survival of specialized herbivores (e.g., Berenbaum, 1981; Scriber, 1984; Tallamy and McCloud, 1991; McCloud et al., 1995). However, it is now becoming clear that many specialist herbivores are negatively affected by small, toxic molecules (Gould, 1988; Baldwin, 1989, 1991; Karban, 1991) and that these defenses can be extraordinarily variable in time and many are strongly and rapidly inducible by specialist herbivore feeding (Baldwin and Ohnmeiss, 1994; Baldwin et al., 1994a,b; Karban and Niiho, 1995; Karban and Adler, 1996).

Here we report the results of field experiments to investigate the effects of highly mobile, toxic plant defenses on the growth and survivorship of a specialist insect herbivore. The monarch butterfly, *Danaus plexippus* L., feeds almost exclusively on milkweeds in the genus *Asclepias* (Ackery and Vane-Wright, 1984; Malcolm and Brower, 1986; Malcolm, 1991, 1995). Both monarch larvae and adults are thought to benefit from host-plant-derived chemical defenses, which they sequester for use in their own defense against natural enemies (Malcolm, 1991, 1995), rather than suffer growth or survival costs associated with these plant chemicals. These defenses include cardenolides, a group of toxic steroids with low molecular weights, that target the ubiquitous Na^+, K^+-ATPase receptor sites of all animal consumers (Horisberger, 1994). Cardenolides occur constitutively throughout the plant and in the white, milky latex characteristic of almost all *Asclepias* species (Malcolm, 1995), and like some other secondary compounds, they are inducible by damage to leaf tissue (Malcolm and Zalucki, 1996).

Early-instar monarchs are known to have variable but generally poor survival (Zalucki and Kitching, 1982a), as is common in the Lepidoptera (Dempster, 1983; Kyi et al., 1991). Based on field observations of naturally laid eggs, Zalucki et al. (1990) showed that survival in the first instar was weakly negatively correlated with plant cardenolide concentration in *Asclepias humistrata* in Florida. Zalucki and Brower (1992) subsequently confirmed this observation with a field experiment. They suggested that some of the high, early-instar losses might be related to the cardenolide concentration in latex or to latex per se. However, observations of larval behaviors by others and ourselves suggest that the larvae are adept at avoiding latex and circumventing the mechanical stickiness and possible toxicity of latex (Brewer, 1977; Rothschild, 1977; Dixon et al.,

1978; Dussourd and Eisner, 1987; Dussourd, 1990, 1993; Zalucki and Brower, 1992). Larvae reduce latex flow by various feeding behaviors to trench or partially sever leaf petioles or veins and then they feed on leaf tissue distal to the trenched or partially severed areas. These behaviors suggested that we could mimic this behavior, and pilot experiments showed that we could reduce latex flow in a similar manner by partially severing the petioles of milkweed plants growing in the field.

Here we investigate further the hypothesis that milkweed defenses influence monarch larval growth and survival. First, we measured latex flow in three common northern species of *Asclepias* found in Michigan, and then we performed field experiments that measured the effect of manipulated latex flow on the growth and survival of first-instar monarchs. The three milkweed species were: *A. syriaca* (common milkweed), which is the major host of the monarch butterfly in the summer part of its range (Malcolm et al., 1989, 1993; Malcolm, 1995), *A. incarnata* (swamp milkweed), and *A. tuberosa* (butterflyweed). These species offer an interesting contrast to the southern *A. humistrata* in that their cardenolide levels are much lower, but latex levels are either comparable (as in *A. syriaca*) or much lower (as in *A. incarnata* and *A. tuberosa*).

METHODS AND MATERIALS

Plant Latex Measurement. Latex production in different milkweeds was compared by regressing latex volume samples against the cross-sectional area of their sources. We measured with calipers the cross-sectional diameter of leaf lateral veins, midrib veins, petiole, and stem at various points on plants so that we could calculate the cross-sectional latex source area. Nonarticulated laticifers ramify throughout stem and leaf tissues (Wilson and Mahlberg, 1980; Dussourd, 1993), and so the cross-sectional area was used as the independent variable against which we regressed latex volume. Latex volume was measured by rapid severance at each cross-sectional point and collection of the flowing latex in volumetric capillary tubes. The cumulative volume produced until flow ceased was measured. Each section was cut from a different plant module (stems or ramets from one or more genets) so that prior damage did not influence latex flow.

Larval Experiments. We used monarch eggs, synchronized to hatch simultaneously, glued on leaf disks of experimental plants as illustrated and described in Zalucki and Brower (1992). Eggs laid by wild-caught monarchs bagged around fresh stems of potted *A. curassavica* plants were removed by using a hole punch and were incubated to the "black-head" stage (just prior to larval emergence) for a day-degree (dd) accumulation of 45 dd (Zalucki, 1982). Disks (7 mm diameter) with eggs about to hatch were then glued onto experimental plants (free of wild monarch eggs) by using a small drop of latex from an adja-

cent stem (not included in the experiment). After three to four days (depending on field temperature for the duration of the first instar), surviving larvae were counted and taken to the laboratory where they were weighed wet, dried in a freeze drier, and then reweighed on a Mettler AT261 DeltaRange balance.

In each experiment, we placed 10 leaf disks on each of 10 control plants and 10 on each of 10 treated plants. The partial petiole severance treatment consisted of one of us (for consistency this was M.P.Z. in each case) reducing the latex flow to all leaves on a plant by notching the underside of the petiole three times across approximately 5 mm with a pair of forceps. We recorded the effectiveness of this technique to reduce latex flow by measuring latex volumes taken from a sample of 10 intact and 10 partially severed leaves on different stems of *A. syriaca* 10 min after partial petiole severance. Latex volumes were recorded by weight on preweighed filter paper and were collected from each intact and partially severed leaf from a point cut across the midrib, 4–5 cm from the leaf apex. Partial petiole severance caused a significant reduction in latex volume for both wet weights ($F_{1,18} = 21.9$, $P < 0.001$) and dry weights ($F_{1,18} = 22.1$, $P = 0.002$). Wet weights of latex were reduced by a mean of 52% (22–60%), and dry weights were reduced by a mean of 71% (49–89%).

All partially severed leaves remained turgid and green and were indistinguishable from intact, control leaves. At initiation of each experiment, we recorded plant height, number of leaf pairs, plant flowering status, and the presence and likely cause of preexisting herbivore damage. Disks bearing eggs were placed on the underside of leaves, with no more than three per leaf. At the final assessment, larvae were counted, collected into labeled vials, and placed on ice. The number of unhatched eggs and the number of dead larvae were recorded.

The number of experiments and variations on this procedure are detailed for each plant species below. When comparing effects of experimental treatments within plant species, we used the wet and dry weights of larvae and the proportion that survived. For comparisons among plant species, we converted weights to weight per day degree (dd, above a development threshold of 11.5°C; see Zalucki, 1982), as growth rate will depend (in part) on temperature, and this necessarily varied among experiments run at different times. Daily maximum and minimum temperatures were recorded locally within 10 km of the plant locations.

Host Plant Species. Asclepias syriaca (common milkweed) is the most abundant milkweed in North America and is common throughout the Great Lakes region of the United States and Canada in old fields, agricultural fields, and by roadsides (Woodson, 1954; Malcolm et al., 1989). Plants grow to approximately 1.25 m and have large, pubescent leaves. The plant grows in large patches as clonal genets within which the separate, unbranched stems are ramets of the same genet connected by underground, rhizomelike roots (Woodson, 1954; Bhowmik and Bandeen, 1976; Polowick and Raju, 1982). We conducted three

experiments with this species: July 8–11 at a small patch (ca. 700 plants) near the university campus in Kalamazoo; July 17–20 and August 5–8, both at adjacent large patches (ca. 120,000 ramets/patch) on the northeast side of Kalamazoo. The first and last dates represent the set-up and harvest dates, respectively. In all experiments we deliberately chose pairs of similar stems that were close together (1.5–62 cm apart; mean = 23 cm). One plant was designated the control and the other was treated by partial petiole severance (see above). In the first two experiments we removed the top of each plant to prevent flower development. In the second experiment we included five extra control plants from which the tops had not been removed. The results from this treatment suggested an effect of removing plant tops; thus, in the third experiment we discontinued this practice.

Asclepias tuberosa (butterflyweed) is the second most common milkweed in southern Michigan and grows mostly in sandy, well-drained soils. It is a small, much less modular milkweed with a rosette of mostly unbranched stems growing from a single vertical, fleshy root. The leaves are small, narrow, and pubescent. We conducted a single experiment from July 18 to 21 at a patch of approximately 100 plants. This milkweed has multiple stems per plant, and hence treatments were allocated to stems within plants. We treated 10 plants with each stem treatment receiving 10 egg disks.

Asclepias incarnata (swamp milkweed) grows in low-lying wet areas. It is a relatively tall milkweed (approximately 1.5 m) with narrow, smooth leaves. Plants may have multiple or single branched stems. We conducted a single experiment on this species from July 22 to 25 at a small patch (ca. 15 plants) near the university campus. Treatments were either allocated to adjacent plants (five pairs) or to stems within plants (one plant with three treatment pairs and another with two).

RESULTS

Plant Latex Measurement. The volume of latex produced as a function of severed cross-sectional area was described by significant linear regressions for each of the three species measured in Michigan (Table 1). For comparison, data for *A. humistrata* (from Malcolm, 1995) are given, and the four species are ranked by level of latex production by using regression slopes. Analysis of covariance among the regressions showed that there was a difference among the four species ($F_{3,66} = 2.9$, $P = 0.04$). There was also a difference among the slopes of latex volume regressed against latex source cross-sectional area for the four species ($F_{3,66} = 15.7$, $P < 0.001$). However, source area did not predict latex volume from untransformed data ($F_{1,66} = 1.2$, $P = 0.27$), and so the data were log-transformed and the ANCOVA was repeated. By using log-transformed data,

TABLE 1. REGRESSION DATA FOR LATEX VOLUME AGAINST CROSS-SECTIONAL AREA SEVERED IN FOUR MILKWEED SPECIES[a]

	Regression data				Area (mm^2)	Volume (μl)	Mean
	Slope	Intercept	r	N	range (x)	range (y)	cardenolide[a]
A. humistrata[b]	9.7[f]	18.5	0.90	13	0.01–26	0.3–380	389
A. syriaca[c]	6.6[f]	62.3	0.87	30	0.07–79	3.8–598	50
A. tuberosa[d]	1.2[g]	0.4	0.94	6	0.02–3.7	0.1–4.2	3
A. incarnata[e]	1.1[g]	10.5	0.62	25	0.20–50	0.1–119	14

[a]Data from Malcolm (1991), cardenolide concentrations were measured in whole leaves. The sample sizes, ranges for x and y variates, and mean plant cardenolide levels (μg cardenolide/0.1 g dry leaf tissue) are also shown. Regression slopes (untransformed data) followed by the same letter do not differ significantly (t-test comparisons of least squares means for log-transformed data, $P > 0.05$); other differences are significant at $P = 0.0001$.
[b]$F_{1,12} = 45.3$, $P < 0.001$ (data from Malcolm, 1995).
[c]$F_{1,29} = 90.0$, $P < 0.001$.
[d]$F_{1,5} = 28.3$, $P = 0.006$.
[e]$F_{1,24} = 14.2$, $P = 0.001$.

source area predicted latex volume ($F_{1,66} = 218.9$, $P < 0.001$), and the latex volumes differed among the four *Asclepias* species ($F_{3,66} = 47.7$, $P < 0.001$), as did the regression slopes ($F_{3,66} = 2.98$, $P = 0.038$, Table 1). Thus, most latex is produced by *A. humistrata* and *A. syriaca*, and least latex is produced by *A. tuberosa* and *A. incarnata.*

The cross-sectional areas of the four plant species varied widely (Table 1), which reflects the overall size differences of the plant stems, petioles, and leaf veins, and so the range of latex volumes varied widely among the four species. These sizes show that *A. syriaca* is by far the largest of the milkweeds with the largest stems and vascular tissue, *A. humistrata* is next, followed by *A. incarnata* and *A. tuberosa.*

The two species with the most latex, *A. humistrata* and *A. syriaca*, also have higher cardenolide contents than the species with the least, *A. incarnata* and *A. tuberosa* (Table 1). However, *A. syriaca* has considerably lower cardenolide levels than the southern *A. humistrata.* During our field trials, we found that *A. tuberosa* produces small amounts of a clear, watery latex and *A. incarnata* produces small amounts of a more viscous, creamy yellow latex, in contrast to the copious white latex produced by *A. humistrata* and *A. syriaca.*

Thus, *A. syriaca* appears to be exceptional in that it has large latex-carrying cross-sectional areas (petioles, side veins, etc.) and produces high latex volumes, but it has characteristically low cardenolide levels. *A. syriaca* is also by far the most modular of the four milkwood species with single individuals commonly comprised of several thousand ramets. We have not compared latex viscosity or flow rate once a laticifer has been punctured. Our observations suggest, how-

TABLE 2. INITIAL CHARACTERISTICS, HEIGHT, AND LEAF NUMBER OF TREATMENT AND CONTROL PLANTS (OR STEMS) WITH SAMPLE REPLICATES (N) IN 5 EXPERIMENTS WITH THREE MILKWEED SPECIES TO ASSESS GROWTH RATE AND SURVIVAL OF FIRST INSTAR MONARCHS

Asclepias species	Exp.	N	Plant height (m, mean ± SD)		Initial leaf number (mean ± SD)	
			Treatment	Control	Treatment	Control
A. syriaca	1	10	1.05 ± 0.12	1.02 ± 0.14	16.6 ± 2.3	16.7 ± 2.2
	2	10	1.00 ± 0.09	1.02 ± 0.09	21.1 ± 3.7	20.0 ± 2.2
	3	10	0.71 ± 0.15	0.76 ± 0.13	16.5 ± 3.4	18.4 ± 3.1
A. tuberosa	1	10	0.58 ± 0.02	0.59 ± 0.03	36.5 ± 9.9	40.9 ± 9.3
A. incarnata	1	10	1.04 ± 0.14	1.01 ± 0.14	34.6 ± 20.0	33.5 ± 9.8

ever, that these properties differ among the species. For example, the latex of *A. humistrata* appears to be nonviscous and flows rapidly to form many small globules depending on the size of the puncture. In *A. syriaca*, the latex is more viscous and oozes out and, at least on the pubescent undersides of leaves, the copious material can spread by attraction from hair to hair. In *A. incarnata* and *A. tuberosa*, the latex fluid is restricted to the immediate area of the puncture.

Larval Experiments. For *Asclepias syriaca*, control and experimental plant stems were similar in height and initial number of leaves (Table 2). Stems were generally close together with mean between-stem distances (±SD) of 8 ± 9 cm, 33 ± 19 cm, and 28 ± 13 cm, for experiments 1, 2, and 3, respectively. Each of the three experiments was performed within an area of approximately 25 m^2 within a patch of *A. syriaca.* Within each of these areas, it is likely that stems or ramets belonged to the same genet. Our growth and survivorship results reflect variation among ramets from the three separate genets. For the purposes of analysis of weights, we excluded larvae in experiment 1 that had gone through to the second instar. The period of the experiment (July 8–11) was particularly warm (more than 45 dd accumulated above the development threshold of 11.5°C), and the larvae developed much more quickly than expected. Furthermore, a storm on the day prior to harvest stripped leaves with damaged petioles from the treated plants, greatly reducing the number of larvae recovered. We therefore compared only the weights of first instars that we recovered. During the second and third experiments all larvae were first instars, having accumulated only 29 dd and 14.3 dd, respectively.

Larvae were larger (both wet and dry weights) on leaves with partially severed petioles in all three experiments (Table 3). During experiment 2 we included five stems whose tops had not been removed. Leaves on these plants were left intact. Larvae were smaller ($P < 0.05$), with a mean wet weight of 132×10^{-5} g

TABLE 3. WET AND DRY WEIGHTS AND GROWTH RATES OF FIRST-INSTAR MONARCHS FED INTACT LEAVES OF *Asclepias syriaca* OR LEAVES WITH PARTIALLY SEVERED PETIOLES

Experiment	Treatment	Larval weight		*t* test	
		Mean ± SE	*N*	*t*	*P*
Wet weight (× 10^{-5} g)					
1	Intact	252 ± 10	29	2.8	0.009
	Severed	324 ± 26	5		
2	Intact	197 ± 11	53	1.8	0.067
	Severed	228 ± 13	47		
3	Intact	62.1 ± 1.9	75	5.8	<0.001
	Severed	85.2 ± 3.7	66		
Dry weight (× 10^{-5} g)					
1	Intact	39.8 ± 1.4	29	2.4	0.022
	Severed	48.8 ± 3.6	5		
2	Intact	34.7 ± 1.7	53	2.0	0.05
	Severed	39.7 ± 1.9	47		
3	Intact	12.1 ± 0.5	75	5.4	<0.001
	Severed	16.3 ± 0.7	66		
Growth rate (10^{-5} g wet weight/dd)					
1	Intact	5.60 ± 0.22	29	2.8	0.009
	Severed	7.20 ± 0.58	5		
2	Intact	6.81 ± 0.36	53	1.8	0.067
	Severed	7.85 ± 0.43	47		
3	Intact	4.35 ± 0.13	75	5.8	<0.0001
	Severed	5.96 ± 0.26	66		

(SE = 15.3, N = 14) on these stems, compared to larvae from stems with either intact or partially severed petioles but with tops removed in experiment 2 (Table 3). During the third experiment we left the tops of all stems intact.

In order to compare larval growth rates among the three experiments, we have expressed larval growth as a rate per day degree. We concentrate here on wet weights only, as the results are essentially the same for dry weights (Table 3). Damaging leaves and topping plants had effects on larval growth rates ($F_{1,285}$ = 34.4 and $F_{1,285}$ = 56.7, P < 0.001, respectively, Table 4). There was no interaction between leaf damage and topping ($F_{1,285}$ = 0.118, NS). Larvae grew fastest on plants with both severed leaves and tops removed and slowest on intact, untopped plants (Table 4). Growth rates did not differ between larvae from topped plants with leaf petioles intact and larvae from untopped plants with severed petioles (Table 4).

For experiments 2 and 3 that were not affected by thunderstorms, survival was generally high, ca. 70%, and unaffected by plant treatment. For experiments

TABLE 4. MEAN GROWTH RATES (MEAN ± SE, *N*) OF FIRST INSTAR MONARCHS (10^{-5} g WET WEIGHT/DAY DEGREE) FED INTACT LEAVES OF *A. syriaca* OR LEAVES WITH PARTIALLY SEVERED PETIOLES FROM PLANTS WITH TOPS REMOVED OR INTACT[a]

Plant	Leaves	
	Intact	Severed
Intact	4.38 ± 0.14[b] (89)	5.96 ± 0.26[a] (66)
Top removed	6.38 ± 0.26[a] (82)	7.79 ± 0.40[c] (52)

[a]Larval growth rates differed significantly between intact and severed leaves (ANOVA $F_{1,285}$ = 34.4, $P < 0.001$) and between topped and intact plants (ANOVA $F_{1,285}$ = 56.7, $P < 0.001$). Means followed by the same letter are not significantly different at $P < 0.05$.

2 and 3 survival was 62 and 72% on petiole-damaged plants and 67 and 73% on plants with intact petioles, respectively. Experiment 1 was conducted in a small, unrelated patch, and survival on the intact plants that were not adversely affected by the storm was 44%. Experiments 2 and 3 were harvested before the end of the first instar. Extrapolation of mean survival rates to the end of the first instar yielded survival levels of 47 and 30%, respectively.

As with *A. syriaca*, control and treatment plants of both *A. tuberosa* and *A. incarnata* had similar characteristics (Table 2). Unlike the results for *A. syriaca*, there was no effect of treatment on wet or dry weights of larvae from *A. incarnata* (ANOVA $F_{1,110}$ = 0.065, Table 5). Partial petiole severance did affect the size of larvae on *A. tuberosa* (ANOVA $F_{1,75}$ = 4.786, P = 0.032). Again, as for *A. syriaca*, larvae fed *A. tuberosa* leaves with partially severed petioles were heavier than those with intact leaves (Table 5). Not surprisingly, mean growth rates (10^{-5} g/dd) were similar on severed (7.6) and intact (7.7) leaves of *A. incarnata* (Table 6), whereas they were different (P = 0.032, *t* test) for *A. tuberosa*, at 7.6 and 6.5 for severed and intact treatments, respectively.

Survival did not differ between severed (63%) and intact (57%) treatments on *A. incarnata*, but was higher on severed leaf plants (55%) than on intact leaf plants (39%) of *A. tuberosa* (one tailed *t*-test on arcsin square root transformed proportions, P = 0.038).

DISCUSSION

Monarch butterflies in North America have been recorded ovipositing, or feeding as larvae, on 27 of the 108 local *Asclepias* species (Malcolm and Brower, 1986), and most evidence suggests that monarchs are *Asclepias* specialists (Ackery and Vane-Wright, 1984). The basis of this specialization is not clear. Milkweeds are noted for containing a group of steroids known as cardenolides, and

TABLE 5. WET AND DRY WEIGHTS AND GROWTH RATES OF FIRST-INSTAR MONARCHS FED INTACT LEAVES OR LEAVES WITH PARTIALLY SEVERED PETIOLES OF *Asclepias incarnata* AND *A. tuberosa*

Experiment and treatment	Larval weight		*t* test	
	Mean ± SE	*N*	*t*	*P*
A. incarnata				
Wet weight ($\times 10^{-5}$ g)				
Intact	247 ± 8.9	55	0.26	0.80
Severed	244 ± 10.4	57		
Dry weight ($\times 10^{-5}$ g)				
Intact	39.7 ± 1.65	54	0.40	0.69
Severed	40.6 ± 1.29	57		
Growth rate (10^{-5} g wet weight/dd)				
Intact	7.66 ± 0.27	55	0.26	0.80
Severed	7.55 ± 0.32	57		
A. tuberosa				
Wet weight ($\times 10^{-5}$ g)				
Intact	166 ± 9.1	33	2.19	0.03
Severed	195 ± 9.0	44		
Dry weight ($\times 10^{-5}$ g)				
Intact	31.9 ± 1.65	33	0.89	0.38
Severed	34.0 ± 1.65	44		
Growth rate (10^{-5} g wet weight/dd)				
Intact	6.51 ± 0.36	33	2.19	0.03
Severed	7.63 ± 0.36	44		

TABLE 6. COMPARATIVE GROWTH RATES OF FIRST-INSTAR MONARCHS REARED ON INTACT LEAVES OR LEAVES WITH PARTIALLY SEVERED PETIOLES OF FOUR SPECIES OF *Asclepias*

Asclepias species	Growth rate (10^{-5} g wet wt/dd	
	Intact	Severed
A. humistrata[a]	3.5	7.8
A. syriaca	4.4	6.0–7.8
A. tuberosa	6.5	7.6
A. incarnata	7.7	7.6

[a]Data from M. Zalucki, L. P. Brower, A. Alonso-M., and T. Van Hook (unpublished data).

some authors have suggested a role for cardenolides in host-plant selection (Brower, 1961; Cohen and Brower, 1982; Zalucki and Kitching, 1982b; Malcolm and Brower, 1986), while others have disputed these findings (Dixon et al., 1978; Zalucki et al., 1989; see Oyeyele and Zalucki, 1990 for a review).

Like many plant secondary compounds these steroids may act as toxins to potential herbivores (Malcolm, 1991, 1995). As the name milkweed implies, plants in this family are noted for their milky latex that is usually rich in cardenolides (Nelson et al., 1981; Seiber et al., 1982; Van Emon and Seiber, 1985). The latex is contained under pressure in a reticulated, sealed system of vessels called laticifers (Lucansky and Cloug, 1986). When a leaf is punctured or its petiole cut, latex flows out and coagulates on contact with air. This system of latex-bearing canals, pressurized with a high concentration of toxic cardenolides in a quick-setting glue, has been interpreted as a plant defense, particularly against generalist herbivores (Dussourd and Eisner, 1987; Dussourd, 1990, 1993).

Monarchs are considered to have evolved the ability to circumvent these physical defenses of milkweeds and subsequently to exploit the chemical defenses of the plant by sequestering them for use against third trophic level enemies (Malcolm, 1991, 1995). Monarch larvae of all stages show various behaviors that may effectively disable the canal-based defenses of milkweeds. Early instars use various forms of vein snipping and trenching (Dussourd, 1990, 1993; Zalucki and Brower, 1992), and late instars can partially sever the petiole before consuming the leaf (Brewer, 1977; Dussourd and Eisner, 1987; Zalucki and Brower, 1992). Monarch larvae feeding on milkweeds concentrate cardenolides above the concentration in the plant (Malcolm and Brower, 1989; Nelson, 1993; Malcolm, 1995) and conserve a mixture of cardenolides through to the adult stage (Brower et al., 1988), possibly as a form of storage excretion in the cuticle. This aspect of monarch biology has attracted considerable attention, particularly with respect to adult cardenolide content and its effect on predation by birds (Brower, 1984; Brower et al., 1988).

More recently, we have investigated the relationship between plant cardenolides, the latex system of milkweeds, and early-instar growth and survival. Most eggs are found on plants with intermediate cardenolide levels (Zalucki et al., 1989; Oyeyele and Zalucki, 1990; van Hook and Zalucki, 1991), and females display postalighting discrimination against plants with low and high cardenolide concentrations (Zalucki et al., 1990). Survival of the resulting first instars to the second instar on various milkweed species is generally poor, ranging from 3.4 to 40% (see Zalucki and Brower, 1992 for a review), and is generally skewed. In field experiments, Zalucki and Brower (1992) found only 3.4–11.5% of newly hatched larvae survived the first instar on *A. humistrata*. Early-stage survival was negatively correlated with plant cardenolide level (see also Cohen and Brower, 1982; Zalucki et al., 1990) and was not affected by ground-dwelling predators. About 30% of larvae were found mired in the leaf latex and glued to the leaf sur-

face. This was despite the elaborate latex-"sabotaging" behavior of first instars. We observed that larvae frequently encountered latex while sabotaging veins and feeding on leaf tissue. During such encounters, latex often adhered to the mouthparts and head, which generally resulted in vigorous cleaning behaviors. The larvae also apparently inadvertently imbibed latex, which generally resulted in catalepsis (as noted in Zalucki and Brower, 1992). As the concentration of cardenolide is much higher in the latex than in leaf tissue (Roeske et al., 1976; Seiber et al., 1982; Zalucki and Brower, 1992), we suspected that this may have been responsible for the catalepsis. However, it was not clear whether cardenolides per se or something else in the latex was the cause of the high mortality that we observed.

Here we investigated the effects of latex on early-instar survival and growth by cutting off latex supply to leaves by partially severing leaf petioles of *A. humistrata* (M. P. Zalucki, L. P. Brower, A. Alonso-Mejia, and T. Van Hook, unpublished data), and *A. syriaca*, *A. tuberosa*, and *A. incarnata* (this study). The damage we inflicted to petioles is like that caused by late-instar monarchs, and two other insect specialists of milkweeds, the cerambycid beetle, *Tetraopes tetrophthalmus*, and the curculionid beetle, *Rhyssomatus lineaticollis*. Our experimental protocol significantly reduced latex flow to leaves (data in Methods and Materials) and had little effect on leaf moisture content. As Zalucki et al. (unpublished data) found with *A. humistrata*, damaging the leaf petiole can increase the growth rate and survival of first instars, but the result differs among milkweed species, possibly reflecting the amount of latex produced and its qualities. Larval growth rate increased on *A. syriaca* with both high latex and low cardenolide when leaf petioles had been damaged or plants had been topped. In either case, we interpret this to reflect reduced latex volume or pressure and greater time available for feeding on such plants because less time is wasted avoiding latex. Unlike *A. humistrata*, survival was unaffected by leaf severing, and this might reflect the generally low cardenolide content of *A. syriaca*. Survival was higher on *A. syriaca* (30–77%) than on any of the other milkweeds reviewed by Zalucki and Brower (1992), and during our experiments we made no attempt to restrict access by predators to experimental plants.

For *A. incarnata*, there was no influence of reduced latex flow on growth rates and survival of first-instar monarchs. This is both a low latex and low cardenolide plant species (Table 1), with smooth, soft, lanceolate leaves. Survival was high (57–63%), even on plants to which predators had easy access. This lack of a response to latex in a low-latex/low-cardenolide milkweed lends weight to the suggested role of both latex and cardenolides as integrated defenses against early-stage monarch larvae.

If this conclusion were generally true among milkweed species, we should have seen the same result with *A. tuberosa*, which is also a low-latex/low-cardenolide milkweed. However, restricting latex in *A. tuberosa* improved both survival

(both 39 to 55%), and growth rates (significantly from 6.5 in intact plants to 7.6 in severed plants; see Table 5). Interestingly, survival was also higher on *A. tuberosa* than on milkweed species with both high latex and high cardenolide (Zalucki and Brower, 1992). Nevertheless, the effect of our plant treatment may reflect other properties of the latex system and its contents in *A. tuberosa.* Only two cardenolide glycosides and their common genin have been isolated from *A. tuberosa* (Petricic, 1966), and it is curious that these particular cardenolides have only been isolated from African *Asclepias* (=*Gomphocarpus*) species and not from other North American *Asclepias* species (Roeske et al., 1976). Possibly, *A. tuberosa* is sufficiently unapparent to ovipositing monarch females that it is not exploited with sufficient frequency to select for defenses against adapted specialists.

There can be no doubt as to the suitability of milkweed leaf tissue for early stage monarch growth. Growth rates of first instars on four species are comparable, once the latex system is circumvented (Table 6). However, both the amount of latex a species produces once a laticifer is ruptured and its cardenolide level adversely affect the growth rate and survival of newly hatched larvae. Once the vulnerable first instar is passed, larvae of this specialized herbivore can probably increase or induce their ability to handle plant chemical and mechanical defenses as they grow larger mouthparts that can more readily trench and overcome the latex system.

Another problem for monarchs on milkweeds was recently raised by Malcolm and Zalucki (1996). Levels of cardenolides are induced above constitutive levels in damaged leaves, and this induction effect occurs rapidly with a peak after 24 hr and then declines over six days. Thus, not only do monarch larvae have to contend with constitutive levels of cardenolide and latex in plants, but they also have to handle cardenolides that are probably rapidly induced in response to feeding. If latex slows larval growth and reduces survivorship, induced cardenolides may also impact growth and survivorship either directly or indirectly by forcing larvae to move to a new leaf. The extensive movements characteristic of early instars among leaves and ramets of milkweeds may be a reflection of this multicomponent defense and a good indication that these "adapted specialists" do indeed incur a cost to feeding on milkweeds.

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INSECTICIDAL EFFECTS OF EXTRACTS FROM TWO RICE VARIETIES TO BROWN PLANTHOPPER, *Nilaparvata lugens*

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Abstract—Ether extracts from a resistant rice variety, Jingxian 89, and a susceptible variety, Qidaizhan, were significantly toxic to *Nilaparvata lugens* adult females, causing 98% and 73.5% mortalities, respectively. The area covered honeydew droplets excreted by *N. lugens* adult females while feeding on Qidaizhan was 28.5-fold more than the area of those feeding on the resistant variety, Jingxian 89. The honeydew covered areas were reduced 29.7- and 8.8-fold, respectively, after feeding on plants treated with the ether extracts from both varieties. Among the fractions from each variety, fractions 9 (three sterols) from both varieties and fraction 10 (3-nitraphthalic acid) from Jingxian 89 showed significant repellent effects on the planthoppers. Further bioassays revealed that fraction 9 from extracts of both varieties at 0.02 mg/ml or higher deterred the feeding activity of the planthoppers, whereas the deterrent effects of fraction 10 from the extract of Jingxian 89 were 100- to 500-fold stronger than those of fraction 9. Fraction 10, identified as 3-nitraphthalic acid, unique in Jingxian 89, was extremely toxic to *N. lugens* adult females, and the LC_{50} and LC_{90} were 0.00045 and 0.00525 mg/ml, respectively. After extensive bioassays and analysis, we concluded that 3-nitraphthalic acid plays a key role in the resistance to *N. lugens* adult females.

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Key Words—Rice, feeding deterrent, brown planthopper, *Nilaparvata lugens* resistance variety, plant resistance.

INTRODUCTION

The brown planthopper, *Nilaparvata lugens* (Stal), is one of the most important pests of rice worldwide (Oercke, 1994). Resistance in rice varieties has been increasingly recognized as the most desirable and economically feasible control tactic in the management of the brown planthopper. However, crop resistance can only be fully utilized after we understand its mechanism. Previous studies have shown that the chemical mechanisms involved with resistance are diverse and may affect the planthoppers' feeding behaviors in various ways, such as plant surface exploration, probing, or ingestion (Sogawa, 1977, 1982). For example, oxalic acid, which inhibits ingestion in *N. lugens*, is twice as concentrated in resistant rice varieties as in susceptible varieties (Yoshihara et al., 1979). The apigenin-C glycosides have been reported as probing stimulants (Kim et al., 1985) and sucking deterrents (Grayer et al., 1994). Stevenson et al. (1996) believed that these compounds are responsible for rice resistance to *N. lugens*.

Our objective in this study was to determine the mechanism of resistance of a resistant rice variety, Jingxian 89 to *N. lugens* (Tan et al., 1995) by comparison with a susceptible rice variety, Qidaizhan, using the extracts to bioassay the toxicological, repellent, and deterrent effects on adult females of *N. lugens*.

METHODS AND MATERIALS

Rice Plants and Insects. A resistant rice variety, Jingxian 89, and a susceptible variety Qidaizhan, were grown in paddy fields in Dinghu District, Zhaoqing City, Guangdong. The plants had been transplanted on April 15, 1995. *N. lugens* adults, collected from rice fields in Dinghu District, were reared on rice variety Qidaizhan in screen cages (35 × 35 × 80 cm) in a laboratory at 25 ± 5°C and relative humidity 80 ± 10%.

Extraction of Rice Plants. Rice plants were sampled from the field when the plants were 61 days old at boot stage. Fresh leaves (2 kg) with both young and old leaves from each of the two varieties were cut into pieces (about 3 cm long). The leaves were immersed in 95% ethanol for two days, and filtered using double-layer paper filter. This procedure was repeated twice, and the ethanol extracts were combined (about 5 liters). The extract was evaporated under reduced pressure by using a standard vacuum pump. The enriched extract was washed three times with petroleum ether (30–60°C). The postether extract was partitioned three times with ethyl acetate. Dry ether extract, postether acetidin extract and postacetidin water extract were obtained by evaporating under reduced pressure for 2 hr and stored in the refrigerator at 4°C.

The ether extract of each variety was subjected to preliminary separation by flash column chromatography using 100 5-cm columns packed with 400 g of 60- to 100-μm preparative silica gel (Qindao Marine Chemical Plant, Qindao, China). Two-thousand gram leaf equivalents (GLE) of the extract were loaded onto the column. One hundred fourteen fractions (100 ml each) were collected by sequentially eluting the column with 2500 ml each of petroleum ether and 20, 40, 60, 80, and 100% ethyl acetate in petroleum ether. The compounds in these fractions were compared using a thin-layer chromatography system (TLC) with five 10-cm, 0.25-mm-thick silica gel (same as above) plates, with petroleum ether–ethyl acetate (4 : 1) as the solvent system. Plates were examined under an iodine-containing box. Those fractions with same or similar migratory rates in the TLC tests were combined to form a new fraction. Thus, 13 and 12 new fractions were obtained from the ether extracts of Jingxian 89 and Qidaizhan, respectively. These fractions were bioassayed for bioactivity on *N. lugens* adults.

Extract Analysis. GC-MS analysis of fractions 9 or 10 was performed on a HP5972 MSD instrument (Hewlett Packard Company) with an electron ionization energy of 70 eV and an ion current of 50 μA using a HP-5 capillary column (50 m × 0.32 mm × 0.17 m). Helium was used as the carrier at a flow rate of 1 ml/min. Samples (1 μl) was injected into the column (Ganeswara-Rao et al., 1995). The column temperature was held at 80°C for 5 min, then programmed at a 3°C/min increment to 290°C.

Toxicological Effects of Extracts. Three 20-day-old Qidaizhan seedlings (five leaves and approximately 10 cm high) were dipped separately for 3 sec in 1 GLE/ml solutions of extract from each variety (diluted in acetone) and placed in a 2000-ml beaker. Seedlings treated with acetone alone were used as controls. Ten 1-day-old *N. lugens* adult females were placed in each of the treated or control beakers for about 30 min after the acetone had evaporated. The mortality was recorded 24 hr after the introduction of the planthoppers. Each treatment was replicated five times, and all tests were conducted at room temperature (25 ± 5°C) and a 12L : 12D photoperiod. Percent mortalities relative to controls were computed according to Zhang (1989): $RM\% = [(TM\% - CM\%)/(100 - CM\%)] \times 100$, where RM is the mortality relative to control; TM is the mortality on treated plants; and CM is the mortality on the untreated plants (control). Percent mortalities were arc sine transformed before analysis of variance (ANOVA) (Gomez and Gomez, 1984), although untransformed mean percentages are reported. Mean percent mortalities were separated using the least significant tests (LSD) (SAS Institute, 1996).

Honeydew Excretion. N. lugens excretes large amounts of honeydew when feeding on the rice plants. However, the amounts of honeydew excreted and the area of the honeydew droplets deposited by the planthoppers vary greatly depending on the developmental stage, host plants, and other biotic and abiotic factors. The planthoppers excrete more honeydew, and the honeydew droplets

cover a larger area, when feeding on preferred host plants or susceptible varieties than when feeding on unpreferred host plants and resistance varieties. The amount of honeydew excreted and the area covered by the honeydew droplets have been used as indirect indicators of host plant preference and resistance (Tan et al., 1995).

Area Covered by Honeydew Droplets. The area covered by honeydew droplets from *N. lugens* adult females after feeding on the plant seedlings was determined as follows, using a feeding chamber (Liu and Zhao, 1996). Ether extracts from each of the two varieties were diluted in acetone. The rice stems were treated with 1 GLE/ml of ether extract from each variety. Control plants were either treated with acetone alone or were untreated. Five 1-day-old adult females were allowed to feed on the rice stems for 24 hr, and the area covered by the honeydew droplets was measured. Each treatment was replicated five times. Areas (square millimeters) covered by honeydew droplets were analyzed, differences between treatments were separated by ANOVA, and means were separated using the LSD test at $P = 0.05$ (SAS Institute, 1996).

Quantity of Honeydew Excreted. The total amount of honeydew excreted over a 24-hr period was measured in the feeding chamber (Hu et al., 1994; Powell et al., 1995). Fractions 9 and 10 from Jingxian 89 extracts and fraction 9 from Qidaizhan extract were recrystallized before the experiment. Artificial diets (10% sucrose + 0.5% glutamic acid) incorporated with either fraction 9 or fraction 10 were used in the bioassay. The amounts of fraction 9 from both rice varieties and fraction 10 from Jingxian 89 in the artificial diets ranged from 0.01 to 1.00 mg/ml, and from 0.002 to 0.00005 mg/ml, respectively. When testing, five 1-day-old *N. lugens* adult females were transferred into the feeding chamber and allowed to feed on a fraction-incorporated artificial diet. Planthoppers feeding on the artificial diets alone were used as controls. *N. lugens* adults were allowed to feed on the diets for 24 hr. The honeydew excreted by *N. lugens* while feeding was collected on the bottom of a plastic flask. After the removal of the adults from the chamber, 2 ml of distilled water was added to the flask to dissolve the honeydew. The solution was stirred for a few seconds and then transferred into a small glass tube. We then added 0.1 ml of 97.1% phenol and 2 ml of 100% sulfuric acid to the tube, and the solution quickly became yellowish brown. After cooling and stirring, the absorbance of the solution at 490 nm was measured. Each treatment was replicated four to five times.

The amount of the honeydew (sucrose equivalent) excreted was calculated using a linear equation, $Y = 3.2209X$, where Y is the amount of the honeydew excretion, and X is the absorbance at 490 nm from a standard curve generated using sucrose. A feeding deterrent index (FDI) (Liu and Zhao, 1996) was used in the rearing experiments and was based on the amount of honeydew excreted. The FDI was calculated as: $FDI = [(HC - HT)/HC] \times 100$, where HC is honeydew in the control, and HT is honeydew in the treatment. The amounts of

honeydew excreted and FDIs were analyzed by ANOVA, and the means were separated using the LSD test as $P = 0.05$ (SAS Institute, 1996).

Numbers of dead and living *N. lugens* adult females were determined after feeding on the treated or untreated plants for 24 hr. Lethal concentrations of fraction 10 extracts for *N. lugens* adult females were calculated by using probit or logit analysis or POLO (LeOra Software, 1994).

Repellent Effects. Three rice seedlings were dipped in appropriate fractions (1 GLE/ml) extracted from each of the two rice varieties. After drying, the seedlings were confined in small cages, and 15 adult females (24 hr old) were introduced into each cage. The location of the planthoppers was examined at 2 hr after introduction. An index based on whether the *N. lugens* adult females were present and feeding on the plant seedlings was calculated using the following formula: $RI(\%) = C/T \times 100$, where RI is the repellent index, C is the surviving *N. lugens* adult females feeding on the seedlings, and T is the total number of *N. lugens* adults tested. Thirteen fractions from Jingxian 89 and 12 fractions from Qidaizhan were tested, and each treatment had five replications.

RESULTS

Extraction of Rice Materials and Analysis. Ether, postether acetidin, and postacetidin water extracts from each rice variety, and a total of 13 and 12 fractions were obtained from the ether extracts from Jingxian 89 and Qidaizhan varieties, respectively. Three major peaks were detected in fractions 9 from Jingxian 89 and Qidaizhan with retention times of 59.05 min (I), 59.70 min (II), and 60.01 min (III). Peaks I, II, and III were then identified as ergosterol, stigmasterol, and β-stigmasterol, respectively. The concentrations of each sterol in the 2 varieties were almost equal according to the peak area. A single major peak was detected in fraction 10 of Jingxian 89 with retention time of 42.37 min (Figure 1), and this peak was identified as 3-nitraphthalic acid. Before this peak, one minor peak was detected with the retention time of 19.07 min, but it has not been identified yet, and its biological and chemical significance is currently unknown.

Toxicological Effects of Extracts. The *RM*'s of *N. lugens* adult females after feeding on the treated plants for 24 hr were significantly different among the three extracts from both Jingxian 89 and Qidaizhan seedlings (Table 1). The percent mortalities caused by the extracts from Jinxian 89 were generally greater than those caused by those from Qidaizhan. However, only the percent mortality for the ether extract from Jingxian 89 (98%) was significantly greater than that from Qidaizhan (73.5%). It is clear that the ether extracts from both varieties have significant insecticidal activities.

Fraction 10 of the Jingxian 89 extract was extremely toxic to *N. lugens*

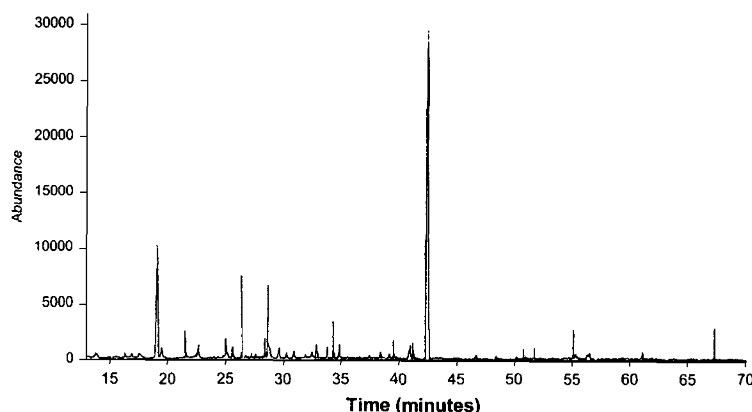

FIG. 1. The chromatogram of fraction 10 from the extract of Jingxian 89. The highest peak was identified as 3-nitraphthalic acid.

adult females (Figure 2). The LC_{50} and LC_{90} were 0.00047 (95% FL: 0.00022–0.00112), and 0.00525 (95% FL: 0.00183–0.11088), respectively.

Area Covered by Honeydew Droplets. The planthoppers fed on the susceptible variety, Qidaizhan, excreted significantly more honeydew than those fed on the resistant variety, Jingxian 89, as indicated by the areas covered by the honeydew droplets after 24 hr of feeding (Table 2). However, the honeydew droplets excreted by *N. lugens* adult females fed on the same susceptible variety (Qidaizhan) seedlings treated with the ether extracts from both varieties decreased dramatically, although the effects of the ether extract from Jingxian 89 were stronger than those of Qidaizhan. The application of acetone alone

TABLE 1. EFFECTS OF THREE TYPES OF EXTRACTS FROM RICE VARIETIES JINGXIAN 89 AND QIDAIZHAN ON *N. lugens* ADULT FEMALES 24 HOURS AFTER TREATMENT[a]

	Mortality (%, mean ± SE)			
Extracts	Jingxian 89	Qidaizhan	*F*	*P*
Ether	97.9 ± 2.0aA	73.5 ± 4.0aB	28.80	<0.001
Post-ether acetidin	18.4 ± 4.5bA	10.2 ± 3.8bA	1.88	0.207
Post-acetidin water	8.0 ± 3.8cA	2.0 ± 2.0cA	0.40	0.545
F	252.93	134.24		
P	<0.001	<0.001		

[a]Means in the same columns followed by the same lowercase letters and in the same row followed by the same uppercase letters are not significantly different ($P = 0.05$, LSD) (SAS Institute, 1996).

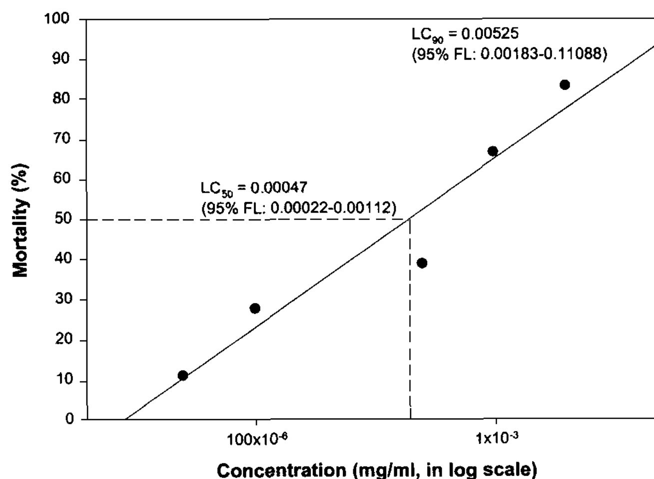

FIG. 2. Toxicity of fraction 10 of extract from Jingxian 89 on *Nilaparvata lugens* adult females after a 24-hr feeding period.

on Qidaizhan did not affect the honeydew excretion or feeding activity of the planthoppers.

Quantity of Honeydew Excreted. The feeding activities of *N. lugens* adult females were significantly affected by feeding on the plant seedlings treated with the fractions (fraction 10 from Jingxian 89 and fraction 9 from both varieties) as indicated by the amount of honeydew (sucrose equivalents) excreted and the FDIs (Table 3 and Figure 3).

TABLE 2. EFFECTS OF ETHER EXTRACTS ON HONEYDEW EXCRETION OF *N. lugens* ADULT FEMALES AFTER FEEDING ON TREATED OR UNTREATED PLANTS FOR 24 HOURS[a]

Treatments	Area covered by honeydew droplets (mm^2/adult, mean ± SE)
Qidaizhan sprayed with ether extract from Jingxian 89	1.86 ± 0.10c
Qidaizhan sprayed with ether extract from Qidaizhan	6.26 ± 0.21b
Qidaizhan sprayed with acetone	57.66 ± 1.85a
Untreated Jingxian 89	1.94 ± 0.21c
Untreated Qidaizhan	55.24 ± 0.78a
F	1030.16
P	<0.001

[a]Means followed by the same letter in the same column are not significantly different ($P = 0.05$, LSD) (SAS Institute 1996).

TABLE 3. DETERRENT EFFECTS OF FRACTION 10 OF JINGXIAN 89 EXTRACT ON *N. lugens* ADULT FEMALES AFTER A 24-HOUR FEEDING PERIOD AS SHOWN BY AMOUNT OF SUCROSE EQUIVALENT[a]

Extract concentration (mg/ml)	Honeydew excreted (μg sucrose/adult, mean ± SE)	FDI
Control	29.12 ± 8.24a	0.00
0.00005	24.86 ± 5.43ab	14.63
0.00010	13.91 ± 2.85bc	52.23
0.00050	3.22 ± 0.76c	88.94
0.00100	3.09 ± 0.92c	89.39
0.00200	2.00 ± 0.75c	93.16
F	7.87	
P	<0.001	

[a]Means followed by the same letter in the same column are not significantly different ($P = 0.001$, LSD) (SAS Institute 1996).

Fraction 10 from Jingxian 89 showed significant feeding deterrent effects on the planthoppers. The amounts of honeydew excreted by the planthoppers after feeding on the seedlings treated with fraction 10 differed significantly among the five concentrations ($F = 7.87$; $df = 5, 24$; $P < 0.001$) (Table 3). The planthoppers excreted significantly less honeydew when feeding on the plants treated with the concentrations of 0.0001 mg/ml or higher. Similarly, the FDIs increased when concentrations of the fraction increased.

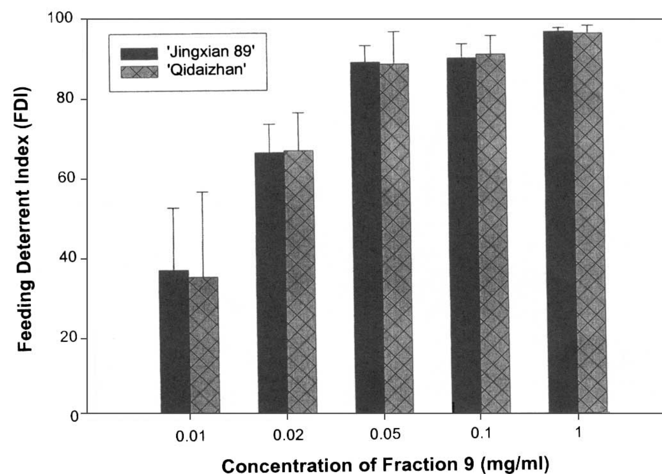

FIG. 3. Feeding deterrent effects of fraction 9 from the extracts of the two rice varieties, Jingxian 89 and Qidaizhan to *Nilaparvata lugens*.

Fraction 9 from both varieties also showed strong feeding deterrent effects on the planthoppers. The FDIs, computed from the amount of honeydew excreted after feeding on the plants, differed significantly between concentrations (F = 9.05; df = 4, 15; $P < 0.001$ for Jingxian 89; F = 3.43; df = 4, 15; P = 0.035 for Qidaizhan) (Figure 3). We consider the extract has feeding deterrent effects if the FDI is 50% or greater. Therefore, fraction 9 caused significant feeding deterrent effects when applied at 0.02 mg/ml or higher. However, the FDIs between the same concentrations of the two fractions from the two rice varieties were not significantly differences ($P > 0.05$).

Repellent Effects. Among the 13 fractions from Jingxian 89, the repellent effects were significantly different (F = 9.48; df = 13, 56; $P < 0.001$), with the strongest effects shown by fractions 9 and 10 (Table 4). The repellent indices among the 12 fractions from Qidaizhan (there is no fraction 10) were also significantly different (F = 5.98; df = 12, 52; $P < 0.001$), with fraction 9 showing a strong repellent effect. All other fractions in both Jingxian 89 and Qidaizhan had no or weak repellent effects on *N. lugens* adult females. These data further support the results from other bioassays, as mentioned above, that the compound, 3-nitraphthalic in fraction 10, which is unique in Jingxian 89, is probably responsible for the resistance of the variety to *N. lugens* adult females.

TABLE 4. REPELLENT EFFECTS OF FRACTIONS FROM JINGXIAN 89 AND QIDAIZHAN ON *N. lugens* ADULT FEMALES AS INDICATED BY REPELLENT INDEX[a]

	Repellent index (%, mean ± SE)	
Fractions	Jingxian 89	Qidaizhan
1	37.3 ± 3.4ab	38.7 ± 3.9ab
2	32.0 ± 2.5abc	34.7 ± 3.9a-d
3	28.0 ± 2.5bc	32.0 ± 5.3a-e
4	25.3 ± 3.9bc	33.3 ± 2.1a-e
5	28.0 ± 3.9bc	29.3 ± 3.4b-e
6	30.7 ± 3.4abc	28.0 ± 2.5cde
7	38.7 ± 4.4a	32.0 ± 2.5a-e
8	28.0 ± 4.4bc	30.7 ± 4.5a-e
9	5.3 ± 2.5d	8.0 ± 1.3f
10	2.7 ± 1.6d	—
11	22.7 ± 1.6c	26.7 ± 4.2de
12	30.7 ± 3.4abc	40.0 ± 3.0a
13	26.7 ± 4.7c	24.0 ± 3.4e
Control	30.7 ± 2.7abc	37.3 ± 1.6ab

[a]Means followed by the same letter in the same column are not significantly different (P = 0.05, LSD) (SAS Institute 1996).

DISCUSSION

Shigematsu et al. (1982) have demonstrated that sterols and asparagine from the rice plants are endogenous factors related to resistance against *N. lugens* by some rice varieties in Japan. As we mentioned above, we found similar compounds (including three sterols) from both rice varieties affecting the feeding behavior of the planthoppers and even causing high mortality. The question is which compound is responsible for the resistance in Jingxian 89. After the extensive bioassays and analyses of the extracts, we now have a better understanding of the resistant variety, Jingxian 89. Firstly, because the ether extracts from both varieties, Jingxian 89 and Qidaizhan, were highly toxic to the planthoppers and strongly deterred the feeding activities of *N. lugens* adult females, as indicated by honeydew excretion, the ether extracts cannot be totally responsible for the resistance in Jingxian 89. Second, three sterols were present in fraction 9 of both varieties with similar concentrations, and both showed significant feeding deterrent and repellent effects on the planthoppers. Therefore, the fraction 9 in Jingxian 89 is not responsible to the resistance to the planthoppers. Finally, 3-nitraphthalic acid is unique in fraction 10 of Jingxian 89, which has great toxicity and feeding deterrent effects against the planthopper. We concluded, therefore, that it is responsible for the resistance to *N. lugens* adult females.

Utilization of resistant rice varieties is one of the major measures in the management of rice pests. Tan et al. (1995) reported that Jingxian 89 is resistant to *N. lugens* but did not reveal its resistance mechanism. The present study quantified the feeding deterrent effects of the extracts and also revealed that 3-nitraphthalic acid may play a key role in the resistance of Jingxian 89 to *N. lugens.*

There are two kinds of resistance, constitutive resistance and induced resistance. Previous studies showed that biological factors (e.g., insects, fungi, bacteria, and viruses) and nonbiological factors (e.g., pesticides) could bring out induced resistance of multiple crops to pests (Lou and Cheng, 1997). If 3-nitraphthalic acid could be introduced into a rice variety or the concentration of the compound could be manipulated and increased, the resistance of the variety to *N. lugens* could be enhanced. At present, we are attempting to obtain purified 3-nitraphthalic acid to verify our results in this study, to conduct further laboratory bioassays and field tests on *N. lugens* and other pests, and to study whether 3-nitraphthalic acid is induced by the feeding activities of *N. lugens.* We also plan to study the function and action mechanism of the compound to *N. lugens* and to determine how the planthoppers adapt to the compound. After finishing these studies, we will be able to better understand the interactions between resistant rice varieties and *N. lugens* and to incorporate the information into programs for breeding resistant rice varieties.

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INFLUENCE OF SYNTHETIC OVIPOSITION PHEROMONE AND VOLATILES FROM SOAKAGE PITS AND GRASS INFUSIONS UPON OVIPOSITION SITE-SELECTION OF *Culex* MOSQUITOES IN TANZANIA

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Abstract—The response of *Culex* mosquitoes to (5*R*,6*S*)-6-acetoxy-5-hexadecanolide (the synthetic oviposition pheromone, SOP), emanations from soakage pit water (SPW), and grass infusions (GI) was studied in pit latrines in Muheza, Tanzania. Water treated with the synthetic oviposition pheromone received more egg rafts of *Culex quinquefasciatus* Say and *Cx. cinereus* Theobald than did the untreated water ($P < 0.001$). The residual activity of SOP did not decrease over a nine-day period, with the geometric mean number of egg rafts laid in the SOP-treated water varying daily between 2.5 and 8.9 as compared to 0.1–1.6 egg rafts laid in tap water. However, SOP did not attract ovipositing mosquitoes to nonbreeding sites, and both treatment and control received few eggs. SPW and GI attracted ovipositing *Cx. quinquefasciatus*, *Cx. cinereus*, and *Cx. tigripes* Grandpré and de Charmoy with the number of eggs rafts deposited in bowls containing SPW or GI being larger than that deposited in bowls with tap water ($P < 0.05$). More egg rafts of *Cx. quinquefasciatus* were deposited in SOP-treated water than in SPW ($P < 0.05$). When SOP was compared with SOP + SPW, more egg rafts of *Cx. quinquefasciatus* were deposited in bowls with the latter combination than in bowls containing SOP or SPW only ($P < 0.05$), indicating a synergistic effect between SOP and SPW. Similarly, when SOP-treated water and GI were compared with SOP + GI, more egg rafts of *Cx. quinquefasciatus* were laid in the latter bowl,

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indicating a synergistic effect between SOP and GI. The results suggest that the combined use of SOP and organically enriched water can be employed in monitoring of *Cx. quinquefasciatus* for control programs. This is the first record of the attraction of *Cx. cinereus* and *Cx. tigripes* to oviposition stimuli.

Key Words—Diptera, Culicidae, *Culex quinquefasciatus*, *Culex cinereus*, *Culex tigripes*, oviposition, behavior, semiochemicals, attractants, Tanzania.

INTRODUCTION

Gravid females of culicine mosquito species show a high degree of preference in selecting specific oviposition sites. This preference may be due to the presence of oviposition pheromones or oviposition attractants and repellents in natural habitats (Bentley and Day, 1989). Oviposition pheromones may occur in nature as intraspecific messengers to inform conspecifics of suitable oviposition sites. Volatiles resulting from the microbial fermentation of organic matter in the aquatic habitat may also function as semiochemicals for gravid mosquitoes to detect suitable or unsuitable oviposition sites (Kline, 1994; McCall and Cameron, 1995).

Oviposition site-selection behavior has been widely studied in *Culex quinquefasciatus* Say (Laurence and Pickett, 1985; Millar et al., 1992). For this species, oviposition is mediated by semiochemicals. In addition to 3-methylindole, which accounts for most of the attraction of female *Cx. quinquefasciatus* to oviposition sites (Millar et al., 1992), numerous other chemical attractants have been identified, and many are associated with high organic or bacterial content (Ikeshoji et al., 1975; Beehler et al., 1994a,b).

Apart from these semiochemicals produced from microbial degradation of organic matter, behavioral observations showed that egg rafts attracted gravid females of *Cx. quinquefasciatus* to oviposit at the same sites. This response is elicited by (5*R*,6*S*)-6-acetoxy-5-hexadecanolide (shortform: acetoxyhexadecanolide), a pheromone released from the apical droplets of 1-day-old eggs (Bruno and Laurence, 1979; Laurence and Pickett, 1982; Laurence et al., 1985). There is increasing evidence that other culicine species also use pheromones released from eggs or larvae as cues for oviposition site location (Takken and Knols, 1999). In a few instances, congeneric *Culex* species respond to the oviposition pheromone of *Cx. quinquefasciatus* (Osgood, 1971).

A synthetic acetoxyhexadecanolide is available and has been used to concentrate egg laying of *Cx. quinquefasciatus* within specific areas in oviposition sites in Kenya (Otieno et al., 1988a). The acetoxyhexadecanolide was formulated in effervescent tablets, which, on contact with water, release the pheromone to the surface of the water, where slow evaporation due to the low vapor pressure of the compound ensured a continuous rate of emission. However, further field

trials of the synthetic oviposition pheromone (SOP) to determine the response of gravid *Cx. quinquefasciatus* in various breeding sites in Africa have not been reported. Moreover, the importance of the oviposition pheromone relative to chemical cues derived from the oviposition site itself or from infusions mimicking oviposition sites has not been determined. The aims of this study were therefore: (1) to determine the response of gravid *Cx. quinquefasciatus* to a SOP in relation to its influence in the selection of oviposition sites in pit latrines; (2) to determine the interaction between SOP and water from natural breeding sites in resource-location behavior of the mosquito; (3) to determine the interaction between SOP and grass infusion (GI); and (4) to assess the period of activity of SOP in the field.

METHODS AND MATERIALS

Study Site

All the experiments were conducted at Ubwari, 5°10′S, 38°46′E, near Muheza, northeast Tanzania. The area lies at an altitude of 200 m above sea level and is about 40 km to the west of the coastal town of Tanga. The mean annual temperature is 26°C, and the average rainfall 1000 mm. The major breeding sites of *Cx. quinquefasciatus* are pit latrines and soakage pits. Most of the pit latrines are filled with water during the rainy season, thus providing optimal conditions for breeding *Culex* mosquitoes. Six pit latrines were selected for the experiments. Each consisted of a slab of concrete about 1 m^2 provided with a drop hole (12 × 25 cm) in the center, suspended over a soakage pit. A small hut of corrugated iron was built surrounding the pit. Access was by a wooden door. The presence of culicine mosquito breeding was confirmed by trapping emerging adult mosquitoes from pit latrines.

Synthetic Oviposition Pheromone

The synthetic oviposition pheromone (acetoxyhexadecanolide) was prepared as described previously (Dawson et al., 1990). Blank effervescent tablets were laid out individually on a clean piece of paper. With a precision syringe, 0.1 ml of SOP solution in hexane (200 mg/ml) was placed on each of the tablets. To prepare the control, 0.1 ml hexane was placed on another effervescent tablet placed on a clean piece of paper. The tablets were left to dry for a few minutes at room temperature before use. For further details see Otieno et al. (1988a).

Experimental Protocol

Each experiment was run in six latrines simultaneously, thus producing six replicates for treatment and control.

Response of Culex *Mosquitoes to SOP.* The first experiment was to assess the response of *Cx. quinquefasciatus* to SOP. Two black plastic bowls (30 cm top surface diameter) were filled with 800 ml of nonchlorinated tap water. An effervescent tablet treated with SOP solution (20 mg/tablet, viz. Otieno et al., 1988a) was placed into one of the bowls, while a hexane-treated tablet was placed in the other bowl as a control. The bowls were placed 1 m apart on the floor of the latrine building at 18:00 hr and left overnight. Each morning, at 08:00 hr, the egg rafts deposited in each bowl were counted and removed. The water was discarded and the bowls rinsed with tap water each day before resetting the experiment. Positions of the treatment and control bowls were alternated each day. The experiment was run for six days.

Residual Activity of Oviposition Pheromone. In order to investigate the duration of activity of the SOP, pheromone-treated bowls were not emptied daily, but only the egg rafts within the bowls were removed and the bowls set for the day's experiment. There were no further additions of SOP or control tablets. The experiment was conducted between 18:00 and 08:00 hr and run for nine days.

Response of Culex *Mosquitoes to SOP, Soakage Pit Water (SPW), and SOP + SPW.* In this experiment, the effect of SPW was at first compared with unchlorinated tap water. In another experiment, SPW, SOP, and their combination were compared in a three-choice arrangement. Water from a soakage pit where mosquitoes were breeding was collected and strained through fine netting to remove all solid debris before being used for the experiment. The bowls containing the treatment and control were placed on the floor of a latrine at 1 m apart. The water in bowls was discarded before setting another day's experiment. Both experiments were conducted between 18:00 and 08:00 hr; the first ran for eight days, while the second ran for six days. In the latter, treatment bowls were alternated daily among the three positions.

Response of Culex *Mosquitoes to SOP, Grass Infusions (GI), and SOP + GI.* GI was prepared by cutting 2 kg of *Digitaria* into small pieces, soaking in 10 liters of unchlorinated tap water in a plastic bucket, and allowing fermentation for five days. The infusion was filtered through fine netting and frozen until needed. In one experiment, six bowls of GI were compared with unchlorinated tap water. The number of mosquito egg rafts deposited was recorded each morning from 800 ml of GI in each of six bowls and the same volume of unchlorinated tap water placed on the floor of a pit latrine building. In another experiment, GI, SOP, and their combination were compared in a three-choice arrangement. This experiment was run for six days. Bowls were treated as in the previous experiment.

Response of Cx. quinquefasciatus *to SOP in a Site Not Normally Supporting Breeding.* Six bowls of tap water were treated daily with the SOP and placed on the verandah of six houses. Six bowls with untreated tap water were used as controls, and each control bowl was placed 1 m from a SOP-treated bowl. The experiment was conducted between 18:00 and 08:00 hr and run for six days.

Data Analysis

In all experiments, the collected egg rafts were sorted by shape and taken to the laboratory and reared separately to the adult stage when they were identified to species (Edwards, 1942; Gillett, 1972). All data were log $(x + 1)$ transformed and means of treatments and controls were compared by using Students t tests. Means in factorial experiments were log $(x + 1)$ transformed and subjected to ANOVA. Any F test significant at $P < 0.05$ was followed by a least significant difference test to compare treatment means.

RESULTS

Apart from the expected presence of *Cx. quinquefasciatus*, *Cx. cinereus* Theobald and *Cx. tigripes* Grandpré and de Charmoy also were found. *Cx. quinquefasciatus* produced pear-shaped egg rafts, *Cx. cinereus* round egg rafts, and *Cx. tigripes* rod-shaped egg rafts.

When the response of mosquitoes to SOP was tested, the bowls containing SOP received more egg rafts of *Cx. quinquefasciatus* and *Cx. cinereus* than did those containing tap water treated with a control tablet ($P < 0.001$, Table 1, A). Except for day 5, more egg rafts of *Cx. quinquefasciatus* were laid in water treated with SOP than in untreated water ($P < 0.05$). The mean number of egg rafts deposited in the pheromone-treated bowls was 4.9 (range 2.5–8.9), with two sites being consistently more attractive than the others. In the control, the mean number of eggs per day was 0.5 (range 0.1–1.6) (Figure 1).

When SPW was compared with unchlorinated tap water (Table 1, B), egg rafts of *Cx. quinquefasciatus*, *Cx. cinereus*, and *Cx. tigripes*, were collected, and for each species the numbers in the treated bowls were different from the control ($P < 0.5$). In a subsequent experiment, the number of egg rafts of *Cx. quinquefasciatus* oviposited in SOP + SPW was different from those deposited in SOP or SPW only, with more egg rafts deposited in the combination treatment ($P < 0.05$). *Cx. cinereus* egg rafts deposited on the SOP-treated water did not differ from those deposited on SPW. However, the number of egg rafts deposited on the SOP + SPW combination was larger than that deposited in either SPW or SOP alone. Although few egg rafts of *Cx. tigripes* were collected in all three treatments, the number of egg rafts deposited in SPW was not statistically different from that deposited in SOP ($P > 0.05$), whereas significantly more egg rafts were deposited on SOP + SPW combination than on SOP or SPW alone ($P < 0.05$) (Table 2, A).

In the comparison of GI with tap water, more egg rafts of *Cx. quinquefasciatus*, *Cx. cinereus*, and *Cx. tigripes* were deposited on GI than on tap water (Table 1, C). When GI was compared with SOP or the SOP + GI combination, the number of egg rafts of *Cx. quinquefasciatus* deposited on SOP was similar

TABLE 1. EGG RAFTS OVIPOSITED BY *Culex quinquefasciatus*, *Cx cinereus*, AND *Cx. tigripes* IN SYNTHETIC OVIPOSITION PHEROMONE-TREATED WATER (SOP) (A), SOAKAGE PIT WATER (SPW) (B), AND GRASS INFUSIONS (GI) (C) VERSUS UNTREATED WATER (CONTROL); $N = 6$ days[a]

	Geometric mean ± SD		
Treatment	*Cx. quinquefasciatus*	*Cx. cinereus*	*Cx. tigripes*
A			
SOP	5.0 ± 0.78a	3.36 ± 0.22a	0
Control	0.18 ± 0.38b	0.43 ± 0.50b	0
B			
SPW	4.84 ± 1.58a	0.17 ± 0.47a	0.53 ± 1.29a
Control	0.28 ± 0.48b	0b	0.12 ± 0.55b
C			
GI	5.64 ± 1.58a	0.31 ± 0.65a	0.28 ± 0.59a
Control	0.24 ± 0.67b	0.02 ± 0.13b	0.04 ± 0.22b

[a] For each of A–C means in the same column followed by a different letter are significantly different ($P < 0.05$).

to that laid on the GI. However, more egg rafts were deposited on the SOP + GI combination ($P < 0.05$), and this number was 2.7 times more than the sum of either treatment alone (Table 2, B). The number of egg rafts of *Cx. cinereus* laid on SOP was larger than that laid on GI ($P < 0.05$), but not significantly different from that deposited on the SOP + GI combination. The mean number of egg rafts of *Cx. tigripes* laid on the three treatments were not different from one another ($P > 0.05$) (Table 2, B).

When the attraction of the mosquitoes to SOP in a nonbreeding area was assessed, no *Culex* egg rafts were found in the SOP-treated bowl at one of the

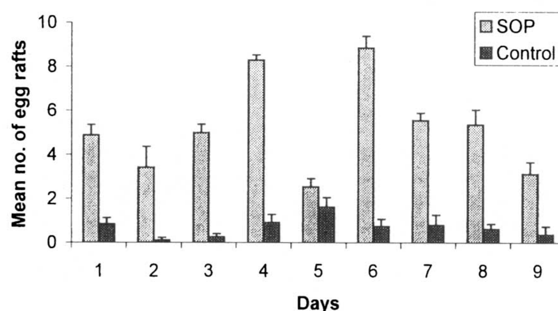

FIG. 1. The residual activity of the SOP (25 mg/liter) expressed as the mean percentage of egg rafts deposited in the treatment and control bowls over time.

TABLE 2. EGG RAFTS OVIPOSITED BY *Culex quinquefasciatus*, *Cx cinereus*, AND *Cx. tigripes* IN SYNTHETIC OVIPOSITION PHEROMONE-TREATED WATER (SOP), SOAKAGE PIT WATER (SPW), THEIR COMBINATION (A), AND IN SOP-TREATED WATER, GRASS INFUSIONS (GI), AND THEIR COMBINATION (B); $N = 6$ DAYS[a]

	Geometric mean ± SD		
Treatment	*Cx. quinquefasciatus*	*Cx. cinereus*	*Cx. tigripes*
A			
SOP	8.6 ± 3.5a	0.36 ± 0.61a	0.06 ± 0.15a
SPW	3.0 ± 1.1b	0.14 ± 0.45a	0.06 ± 0.2a
SOP+SPW	31.51 ± 2.1c	1.74 ± 1.13b	0.32 ± 0.60b
B			
SOP	11.02 ± 2.28a	2.51 ± 1.69a	0.95 ± 1.21a
GI	7.55 ± 1.59a	0.56 ± 0.74b	1.38 ± 1.31a
SOP+GI	49.7 ± 1.87b	1.90 ± 1.36a	1.21 ± 1.0a

[a]For each of A and B, means in the same column followed by a different letter are significantly different ($P < 0.05$).

locations and only a few were found in other locations. The overall oviposition of *Cx. quinquefasciatus* in the SOP-treated water was not different from that in the control bowls ($P > 0.05$), with geometric mean value (±SD) of 0.6 ± 1.0 egg rafts for the former and 0.3 ± 0.5 for the latter.

DISCUSSION

We observed that the water treated with SOP received more egg rafts of *Cx. quinquefasciatus* and *Cx. cinereus* than did the control. This is the first time that *Cx. cinereus* is reported to respond to SOP. In nature, *Cx. quinquefasciatus* and *Cx. cinereus* have been observed to breed in similar habitats in Kenya, Nigeria, and Tanzania (Subra and Dransfield, 1984; Irving-Bell et al., 1987; Lyimo and Irving-Bell, 1988; Mboera et al., 1997). It is likely that the two species respond to similar cues while selecting their oviposition site. Previously, it has been reported that other *Culex* species, such as *Cx. tigripes* Coquillet (Hwang et al., 1987) and *Cx. pipiens molestus* Forskål (Sakakibara et al., 1984), also respond to *Cx. quinquefasciatus* pheromone, and it has been suggested that the pheromone is active in conspecifics of *Cx. quinquefasciatus* (Millar et al., 1994).

Cx. tigripes appeared in breeding sites of *Cx. quinquefasciatus* and *Cx. cinereus* towards the end of the rainy season. Larvae of *Cx. tigripes* are known to prey voraciously on larvae of other mosquito species, such as *Anopheles gambiae* Giles (Lyimo, 1993) and *Cx. quinquefasciatus* and *Cx. cinereus* (L. E. G. Mboera, unpublished data). To our knowledge, this is the first time that *Cx. tigripes* has been observed to respond to olfactory cues. Thus, oviposition cues,

similar to those attracting *Cx. quinquefasciatus* and *Cx. cinereus*, will lead *Cx. tigripes* to breeding sites of its prey. Several predatory mosquito species, e.g., *Toxorhynchites* spp., have been attracted to oviposition site water-borne semiochemicals to which *Aedes* spp. are also responsive (Linley, 1989). Therefore, it is possible that the observed phenomenon is a common strategy for predaceous mosquitoes to locate their prey.

It was initially surprising to find that the residual activity of the synthetic oviposition pheromone did not decrease over a nine-day period. As the total number of egg rafts laid varied greatly among days, with a constant trend between latrines (data not shown), the activity of the pheromone must have been above the optimum threshold (Millar et al., 1994) during the experimental period. It appears worthwhile to extend the duration of this experiment in order to study the actual duration of the oviposition pheromone under natural conditions. As we did not change the pheromone-treated water in the bowls, it is possible that the two treatments were daily complemented by natural pheromone originating from the freshly deposited egg rafts. However, the relatively large dose of SOP together with the inherently low vapor pressure of this compound is known under other, but related, conditions to give a level dose–response relationship once the dose is high enough to achieve the saturated vapor pressure (Laurence and Pickett, 1985), as could be happening here.

The three *Culex* species showed a stronger preference to oviposit on SPW than on unchlorinated tap water. It is likely that this polluted water from a natural mosquito breeding site contains volatile cues used by gravid *Culex* mosquitoes. When SOP-treated water was compared with SPW and SOP + SPW, more egg rafts of *Cx. quinquefasciatus* were laid in the latter treatment, indicating a synergistic effect. A similar phenomenon was observed when GI and SOP were compared with their combination. In two other independent laboratory studies on the effect of SOP plus polluted water (Blackwell et al., 1993) and SOP plus a synthetic mixture of oviposition attractants (Millar et al., 1994), an additive effect, but no synergism, of the combination was found. In the field, it is unlikely that SOP will act independently, i.e., without other indicators of oviposition site suitability. Such indicators are probably more important in the initial selection, with the oviposition pheromone operating subsequently in an augmentative role, as the former are unlikely to be species-specific. Factors other than the oviposition pheromone are, therefore, involved. These factors were not determined in this study, but numerous oviposition attractants have been identified, and many are associated with high organic or bacterial content (Millar et al., 1992; Beehler et al., 1994a). Among other substances found to be attractive to ovipositing *Culex* mosquitoes are fatty acids, as well as compounds produced by *Pseudomonas* and *Aerobacter* bacteria (Ikeshoji et al., 1967, 1975).

The finding that in a nonbreeding area SOP alone did not stimulate oviposition of *Cx. quinquefasciatus* was unexpected, because in a recent study (L. E.

G. Mboera et al., unpublished data) it was found that this mosquito is attracted to SOP from a distance. Initially, attraction over a distance of 5.5 cm (Laurence and Pickett, 1982) and then up to 10 m (Otieno et al., 1988a) was evidenced, and more recently upwind anemotaxis mediated by the SOP has been demonstrated (Pile et al., 1991, 1993). Our combined results, with increased oviposition in SOP-treated bowls inside latrine buildings, suggest a strong interaction in oviposition-inducing behavior between water-derived semiochemicals present in natural breeding sites and the oviposition pheromone. However, it is difficult to determine the relative importance of the pheromone versus other stimuli (such as synergistic semiochemicals, physical and/or visual stimuli) to the overall oviposition behavioral response (Bentley and Day, 1989). To date, little information is available as to the modes of action of substances that mediate oviposition. Appropriate experimental design for differentiating attractants and stimulants as well as for exploring the interplay between these factors is important.

SOP, GI, and SPW clearly elicited strong oviposition responses in field populations of gravid *Cx. quinquefasciatus*, whereas oviposition by *Cx. cinereus* was moderate to the SOP and GI, and low in response to SPW. Our results are in agreement with previous observations that gravid *Cx. quinquefasciatus* oviposits readily in response to SOP in the laboratory and field (Laurence and Pickett, 1982; Otieno et al., 1988a,b). SPW and GI, such as those used in our study, consist of complex mixtures of compounds that vary over time. It has been established previously that some of the oviposition attractants from such organic infusions are products of bacterial degradation, and it is, therefore, not surprising that *Cx. quinquefasciatus* breeds more profusely in polluted water than in clean water. In Tanzania, these stimuli are present in natural breeding sites. In addition, oviposition pheromone and GI or SPW together provide a stronger oviposition stimulus than individual components alone. Oviposition semiochemicals can, therefore, be employed in odor-baited traps for the surveillance and control of *Cx. quinquefasciatus*, an important vector of urban bancroftian filariasis.

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ELECTROPHYSIOLOGICAL AND BEHAVIORAL RESPONSES OF THE CABBAGE MOTH TO PLANT VOLATILES

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Abstract—Plant volatiles from cabbage and chrysanthemum were studied as to how they affect behavior of the cabbage moth, *Mamestra brassicae* (L.). Chemical, electrophysiological, and behavioral techniques were used. The electroantennographic (EAG) evaluation of selected compounds from Brassicaceae showed that isothiocyanates (NCS) elicited weak responses, and some did not evoke significant EAG responses at all. Green leaf volatiles (GLVs) evoked the strongest responses in both male and female antennae. The capacity of NCS to stimulate upwind flight of mated females was not different at doses of 10^{-7}, 10^{-6}, or 10^{-5} g when tested in a wind tunnel. At the higher doses, allyl NCS stimulated upwind flight in the females more than the other compounds. Allyl NCS was significantly better than the other compounds at stimulating females to land on targets. Mated females flew upwind and landed on the targets with allyl NCS more often than virgin females and males. With respect to the behavioral activity of GLVs, only (*E*)-2-hexenal and (*Z*)-3-hexenyl acetate elicited upwind flight and landing in females. Ten compounds were identified from a chrysanthemum extract by using coupled gas chromatography–electroantennography. Five of these, (*Z*)-3-hexenyl acetate, 1-8-cineole, α-terpinene, chrysanthenone, and camphor, elicited upwind flight of mated females, but only three stimulated landing.

Key Words—*Mamestra brassicae*, host-finding behavior, upwind flight, plant volatiles, chrysanthemum, cabbage, isothiocyanates, green leaf volatiles, electroantennographs.

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INTRODUCTION

The cabbage moth, *Mamestra brassicae* (L.) is a nocturnal and polyphagous species. Its known food plants include more than 70 species from 22 families. Species of the Brassicaceae and Chenopodiaceae families are among the most preferred (Popova, 1993). Bretherton et al. (1979) reported that *M. brassicae* is particularly associated with cruciferous crops. Alternative hosts include lettuce, beet, onion, potato, pea, tomato, chrysanthemum, apple, beech, and oaks (Carter, 1984). Recently, the behavioral responses of *M. brassicae* mated females to different host plants were investigated under laboratory conditions by using a wind tunnel (Rojas-León, 1997). In two-choice tests, females initially flew upwind and landed preferentially on tomato and chrysanthemum, compared to cabbage plants, but these same females oviposited more often on cabbage plants.

Chemical identification of semiochemicals mediating host-plant location in phytophagous moths are scarce in comparison to those involved in mate-finding behavior (Honda, 1995). In the case of polyphagous moths, only three studies have identified the plant volatiles mediating female long-range responses to host plants. Orientation of *Trichoplusia ni* (Hübner) females towards a susceptible soybean variety was associated with the volatiles 4-hexen-1-ol acetate, 2,2-dimethylhexanal, and 2-hexenal (Liu et al., 1988). However, no behavioral assays of the separate or combined constituents identified from the attractive extracts were carried out. Pentan-1-ol, (+)-Δ-3-carene, ($\pm$)-α-pinene, and myrcene were identified from chickpea plants (*Cicer arietinum* L.) as kairomones mediating the orientation of mated *Helicoverpa armigera* Hübner females (Rembold et al., 1991). Recently, Hartlieb and Rembold (1996) reported that females of *H. armigera* were attracted by a steam distillate from pigeonpea plants (*Cajanus cajan* L.). A blend of six sesquiterpene hydrocarbons: β-caryophyllene, α-humulene, α-guajene, α-muurolene, γ-muurolene, and α-bulnesene accounted for the activity of the extract. Of these compounds, only α-bulnesene was attractive by itself, and its activity was less than the mixture.

This study was undertaken to investigate the putative role that host-plant volatiles from two species play in host-finding behavior of the cabbage moth. Chemical, electrophysiological, and behavioral techniques were employed.

METHODS AND MATERIALS

Biological Material. The laboratory colony was started with adults collected from a light trap at Tackley, Oxfordshire, during June 1995, and wild insects were added to the colony every summer. All insects were reared in controlled conditions under a 16L : 8D photoperiod cycle at 25 ± 1°C and 30–50% relative humidity and fed on an artificial diet (Rojas and Wyatt, 1999). The

rearing technique is described in Rojas (1999a). Chrysanthemum plants (variety Yuba) were bought locally.

Plant Extracts. Extracts were made by using microwave-assisted extraction. Thirty grams of leaf material were placed in a flask in a modified microwave oven (Craveirs et al., 1989) for 60 sec (medium power level). Volatiles distilled from the plant material were swept from the flask with a stream of nitrogen (600 ml/min) and trapped in a 100-ml flask containing 50 ml of cooled (−5°C) redistilled hexane. Extracts were dried with $MgSO_4$, filtered, and concentrated to 3 ml with a rotary evaporator.

Chemicals. Fourteen volatile compounds reported to occur in Brassicaceae and nine compounds identified in the chrysanthemum extracts were selected for evaluation. All compounds were obtained from commercial sources (Table 1) except 3-butenyl and 4-pentenyl isothiocyanate (NCS) and chrysanthenone. Standard solutions (10^{-4}–10^{-7} g/10 μl) of all compounds were prepared in hexane for EAG and wind tunnel studies.

Chemical Analysis. Gas chromatography (GC) was performed on a Hewlett-Packard model 5880A gas chromatograph fitted with a 50-m × 0.32-mm-ID methyl silicone bonded-phase fused silica capillary column (HP-1), a split/splitless injector, and a flame ionization detector (FID). The carrier gas was hydrogen. The oven temperature was maintained at 40°C for 5 min and then increased at 5°C/min to 150°C, then at 10°C/min to 250°C.

Coupled gas chromatography–mass spectrometry (GC-MS) was performed on a Hewlett Packard 5890 GC coupled to the MS and integrated data system (70–250 VG Analytical). Ionization was by electron impact at 70 eV, 230°C. A HP-1 methyl silicone column (50 m × 0.32 mm ID) was temperature programmed from 30°C (held for 5 min) to 180°C at 5°C/min. Identifications were confirmed by comparison of the mass spectral data with those of authentic samples and by GC peak enhancement (Pickett, 1990).

Electroantennography (EAG). Antennal receptivity of *M. brassicae* females and males to the selected compounds was determined by EAG. An antenna was excised at its base, and the distal part of the terminal segment was cut off. The antenna was mounted between two glass capillary electrodes. The capillaries were filled with saline solution (Blight et al., 1995) into which Ag–AgCl wires were inserted. The signals generated by the antenna were passed through a high-impedance amplifier Syntech UN-06 (Syntech NL 1200, Hilversum, The Netherlands) and displayed on a monitor that used software (Syntech) for processing EAG. Linalool (10^{-6} g) was used as a standard for normalizing all responses so that responses within an individual and between individuals could be compared (Payne, 1975). Stimulation with the standard preceded and followed every two test stimulations.

Stimulus Delivery. The odor delivery system was similar to that previously described (Blight et al., 1995). The stimulus (1-sec duration) was delivered into a

TABLE 1. SOURCE AND PURITY OF COMPOUNDS EVALUATED IN BEHAVIORAL AND ELECTROANTENNOGRAM ASSAYS

Compound	Sample chemical purity (%)	Source
Isothiocyanates		
Allyl NCS	95	Aldrich Chemical Co. Ltd
Butyl NCS	99	Aldrich Chemical Co. Ltd
Benzyl NCS	98	Aldrich Chemical Co. Ltd
3-Butenyl NCS	98	IACR-Rothamsted*
4-Pentenyl NCS	97	IACR-Rothamsted*
2-Phenylethyl NCS	97	Fluka Chemie AG
Green leaf volatiles		
1-Hexanol	99	Sigma Chemical Co. Ltd
(*Z*)-3-Hexen-1-ol	98	Aldrich Chemical Co. Ltd
Hexanal	98	Aldrich Chemical Co. Ltd
(*E*)-2-Hexenal	98	Simga Chemical Co. Ltd
(*Z*)-3-hexenyl acetate	98	Sigma Chemical Co. Ltd
Others		
Methyl salicylate	98	Aldrich Chemical Co. Ltd
Caryophyllene	99	Fluka Chemie AG
Linalool	97	Aldrich Chemical Co. Ltd
1,8-Cineole	98	Fluka Chemie AG
Chrysanthenone	—	IACR-Rothamsted*
Camphor	96	Aldrich Chemical Co. Ltd
Verbenol	95	Fluka Chemie AG
Borneol	98	Fluka Chemie AG
α-Terpinene	97	Aldrich Chemical Co. Ltd
α-Terpinolene	—	IACR-Rothamsted†

*Synthesized by A. Hick.
† Gift from Professor F. Camps, University of Barcelona, Barcelona, Spain.

purified airstream (1 liter/min) flowing continuously over the preparation. Samples (10 μl) of the standard solutions of test compounds were applied to filter paper strips, and the solvent was allowed to evaporate (20 sec). The paper strip was placed in the cartridge or clean pipet and left for 40 sec before applying. The vapor from the cartridge was injected into the airstream passing over the antennal preparation by means of a second airstream. The latter was controlled by using a solenoid valve which was activated by an electronic timer.

Gas Chromatography–Electroantennography (GC-EAG). GC-EAG studies were carried out to locate electrophysiologically active components in the chrysanthemum extract. The system used was previously described by Wadhams (1990). The capillary column effluent was split 1 : 1 between the flame ionization detector (FID) and the transfer line to the insect preparation by a low volume splitter made from glass-lined, stainless-steel tubing and deactivated fused silica tubing. One length of tubing went to the FID while the other was taken through

the oven wall and conducted the rest of the effluent into a purified airflow passing continuously over the antennal preparation. The outputs of the FID and the EAG were simultaneously recorded and displayed on a computer screen by using the GC-EAD software developed by Syntech.

Wind-Tunnel Assays. Assays were carried out in a wind tunnel, 160 cm long and 75 cm high and wide, as described by Rojas and Wyatt (1999). A Fischbach speed controller fan (contained in a detachable cabinet) was used to push air through the tunnel with a velocity of 35 cm/sec. An activated charcoal filter cleaned the air at the intake. Illumination was provided by two 85-W red fluorescent tubes (Thorn EMI, UK), mounted 5 cm above the wind tunnel. The illumination, measured at the point of release 20 cm from the downwind screen and about 45 cm from the tunnel floor, was 4.54 $\mu E/m^2$. A stationary canvas belt, with alternating black and white dots, located immediately beneath the tunnel floor provided optomotor feedback for the moths.

The insects were placed individually into a pot 5 cm high × 4 cm ID (release cylinder) and allowed to acclimate to the conditions in the wind tunnel room for at least 1 hr prior to use. Wind-tunnel observations were carried out with nonchoice tests. A cotton wool wick, loaded with 10 μl of the test compounds or solvent (control), was placed at a height of 30 cm in the center of the upwind end of the tunnel. Each observation was begun by placing the release cylinder on a 45-cm-high platform at the downwind end, and one insect was released and observed for 3 min. Occasionally, the insects' responses to cabbage or chrysanthemum plants were recorded as controls. Mated females were used for most experiments, except when comparisons were made between mated and virgin females and virgin males to allyl NCS. Data were recorded for zigzagging upwind flight towards a target and for landing following such zigzagging flight. Zigzagging flight was recorded only when females progressing upwind flew to within 5 cm of the target.

Statistical Analysis. Data were analyzed by using the Minitab statistical package. Data were subjected to Bartlett's test for homogeneity of variances, and square-root transformed if required before analyzing with one-way analyses of variance (ANOVA) or a t test assuming equal variances. When data presented zero values, 0.5 was added to the original data before analysis. Original data are shown in the figures. Means separations were accomplished with a Duncan test at $P < 0.05$.

RESULTS

Electroantennogram and Behavioral Responses to Selected Brassicaceae Volatiles

The mean EAG responses of female and male *M. brassicae* antennae were not significantly different from one another for either the hexane control

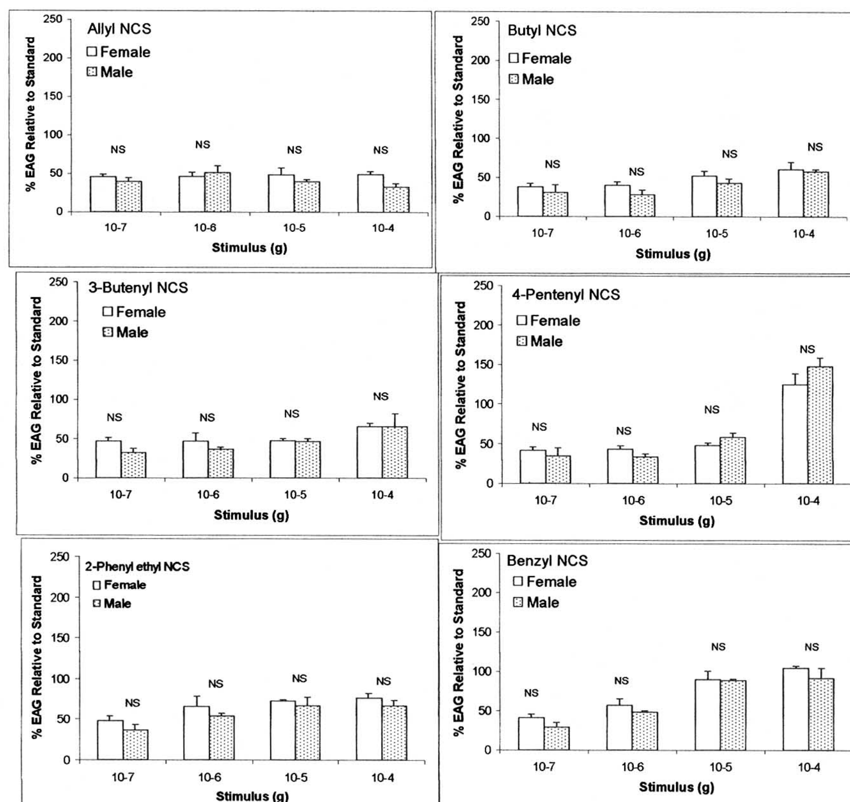

FIG. 1. Mean (± SE) EAGs of female and male *M. brassicae* to different doses of isothiocyanates. NS = no significant sexual difference, $P < 0.05$, *t* test assuming equal variances.

(females, $X \pm SE = -0.18 \pm 0.02$ mV; males, $X \pm SE = -0.23 \pm 0.01$ mV, $N = 20$) and linalool standard (females, $X \pm SE = -1.33 \pm 0.06$ mV; males, $X \pm SE = -1.44 \pm 0.06$ mV, $N = 25$).

Isothiocyanates (NCS). For NCS evaluated, there was a dose effect on the EAG response, except for allyl NCS, for both mated females ($F = 0.28$, $P = 0.597$) and male ($F = 2.29$, $P = 0.146$). There were no differences between male and female responses to any of the NCS compounds (Figure 1). EAG responses for allyl and butyl NCS were not different from the hexane control response at any level. The rest of the NCS evaluated only evoked significantly different EAG responses at 10^{-5} and 10^{-4} g levels. 4-Pentenyl NCS elicited the strongest EAG responses.

The behavioral responses of *M. brassicae* females to NCS evaluated in the

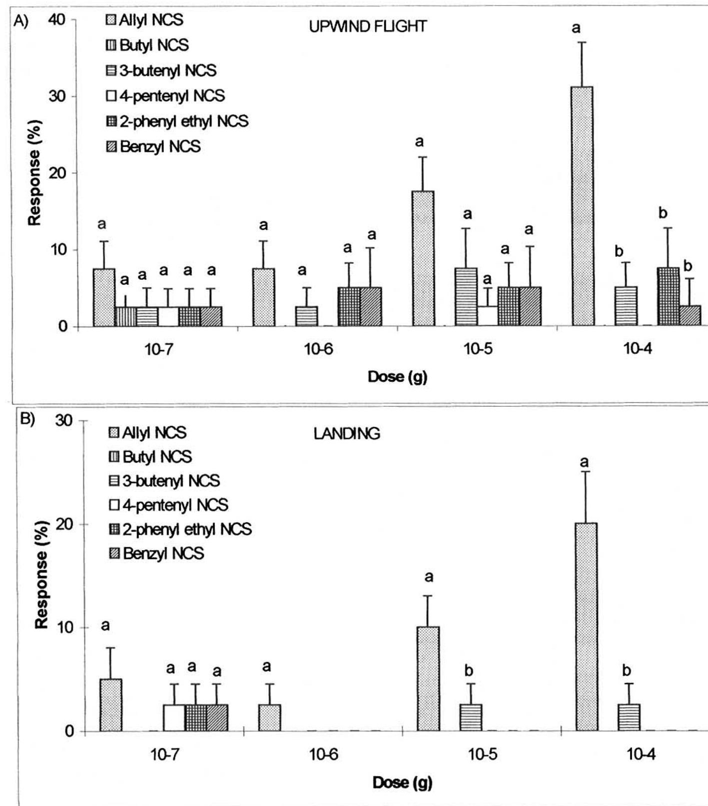

FIG. 2. Behavioral responses ($X \pm SE$) of *M. brassicae* females to isothiocyanates at four doses in the wind tunnel. (A) upwind flight; (B) landing. Data are averages of eight replications; in each, five insects were assayed. Bars marked by the same letter within doses are not significantly different ($P < 0.05$).

wind tunnel are shown in Figure 2. The capacity of these compounds to stimulate upwind flight was not different at 10^{-7} ($F = 0.53$, $P = 0.748$), 10^{-6} ($F = 0.87$, $P = 0.508$), or 10^{-5} g ($F = 1.89$, $P = 0.133$). At 10^{-4} g, allyl NCS stimulated females more than the other compounds ($F = 5.52$, $P = 0.001$). All NCS evaluated, except butyl and 3-butenyl, elicited few landings at the 10^{-7} g level. At the higher doses, only 3-butenyl and allyl NCS stimulated females to land on target. Allyl NCS was better than 3-butenyl at the 10^{-4} g ($t = 3.20$, $P = 0.003$) dose (Figure 2).

Mated females flew upwind ($F = 7.04$, $P = 0.004$) and landed ($F = 5.68$, $P = 0.010$) on the targets with allyl NCS (10^{-4} g) more often than virgin females or males (Figure 3).

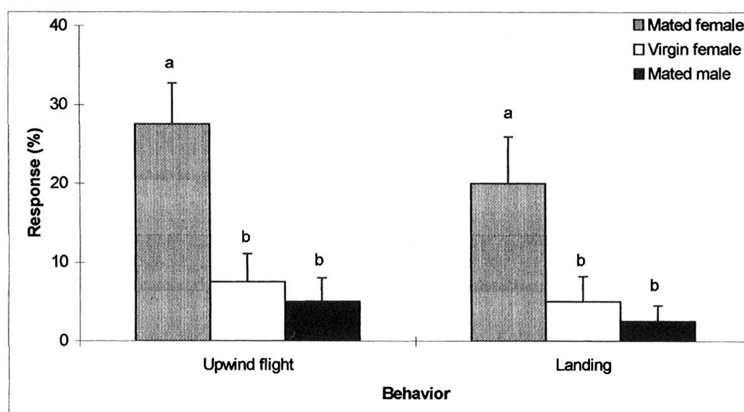

FIG. 3. Mean percentage (± SE) responses of mated and virgin females, and virgin males to allyl NCS (10^{-4} g). Data are averages of eight replications; in each, five insects were assayed. Bars marked by the same letter are not significantly different ($P < 0.05$).

Green Leaf Volatiles (GLVs). For the five green leaf volatiles evaluated, there was a dose effect on the EAG response of mated male and females antennae, except for the male response to (Z)-3-hexenyl acetate ($F = 4.24$, $P = 0.054$) (Figure 4). There were also differences between male and female responses to all compounds of at least one dose level (Figure 4). When a sexual difference was observed, the female response usually was higher, except with 1-hexanol and (Z)-3-hexenyl acetate. Of the compounds evaluated, 1-hexanol elicited the greatest EAG response (Figure 4).

The five green leaf volatiles elicited EAG responses compared to a hexane control at the 10^{-5} and 10^{-4} g level, although in the case of (Z)-3-hexan-1-ol, 1-hexanol, and (Z)-3-hexenyl acetate, these responses were obtained at the 10^{-6} g level with female antennae.

With respect to the behavioral activity, only (E)-2-hexenal and (Z)-3-hexenyl acetate elicited upwind flight and landing in females (Figure 5). There was no difference between these two compounds in stimulating upwind flight (10^{-7}: $t = -0.62$, $P = 0.274$; 10^{-6}: $t = -0.62$, $P = 0.274$; 10^{-5}: $t = -0.54$, $P = 0.299$; 10^{-4} g: $t = -1.11$, $P = 0.144$) or landing (10^{-7}: $t = -0.62$, $P = 0.274$).

Other Compounds. There was an effect of dose on the EAG response of female and male antennae to both caryophyllene (female: $F = 28.16$, $P < 0.001$, male: $F = 8.84$, $P = 0.008$) and methyl salicylate (female: $F = 26.29$, $P < 0.001$; male: salicylate ($F = 21.67$, $P < 0.001$) (Figure 6). There was no difference between male and female responses to methyl salicylate, but the female antennae showed a stronger response to caryophyllene (Figure 6). Methyl salicylate

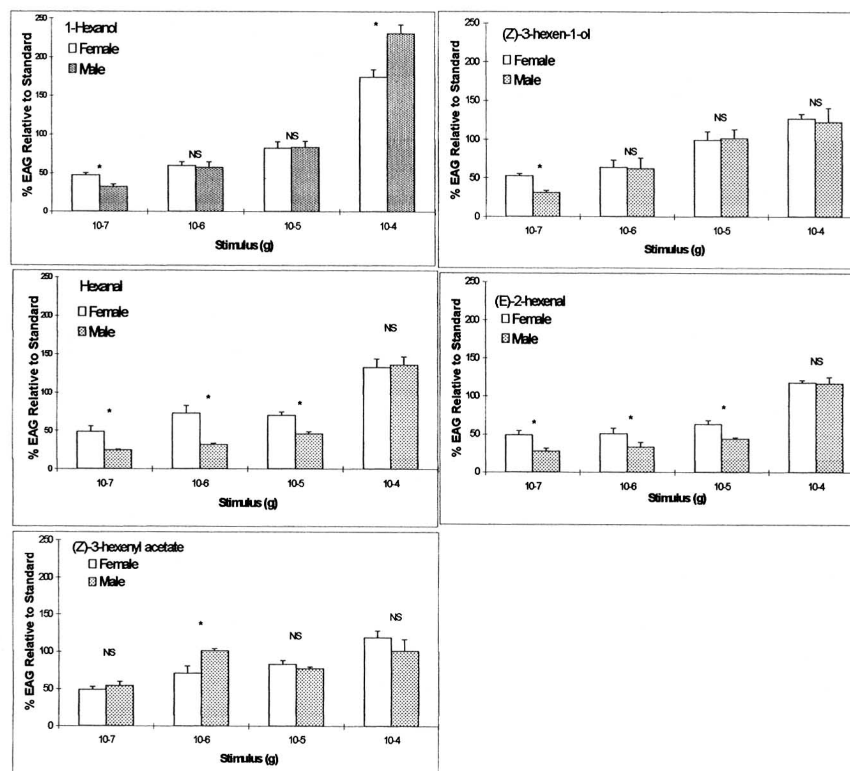

FIG. 4. Mean (± SE) EAGs of female and male *M. brassicae* to different doses of green leaf volatiles. NS = no significant sexual difference; *significant sexual difference, $P < 0.05$, t test assuming equal variances.

elicited EAG responses compared to a hexane control at any level with both male (10^{-7}: $t = 2.29$, $P = 0.025$; 10^{-6}: $t = 4.91$, $P < 0.001$; 10^{-5}: $t = 13.31$, $P < 0.001$; 10^{-4} g: $t = 10.18$, $P < 0.001$) and female (10^{-7}: $t = 2.29$, $P = 0.02$; 10^{-6}: $t = 3.68$, $P = 0.003$; 10^{-5}: $t = 6.94$, $P < 0.001$; 10^{-4} g: $t = 15.89$, $P < 0.001$) antennae. Caryophyllene elicited EAG responses with respect to the control at 10^{-5} ($t = 4.23$, $P = 0.001$) and 10^{-4} g ($t = 5.65$, $P < 0.001$) in male; and at 10^{-6} ($t = 2.89$, $P = 0.01$), 10^{-5} ($t = 8.64$, $P < 0.001$), and 10^{-4} g ($t = 10.45$, $P < 0.001$) in female antennae.

The evaluation of caryophyllene and methyl salicylate in the wind tunnel showed that these compounds did not stimulate upwind flight or elicit landing behavior on targets.

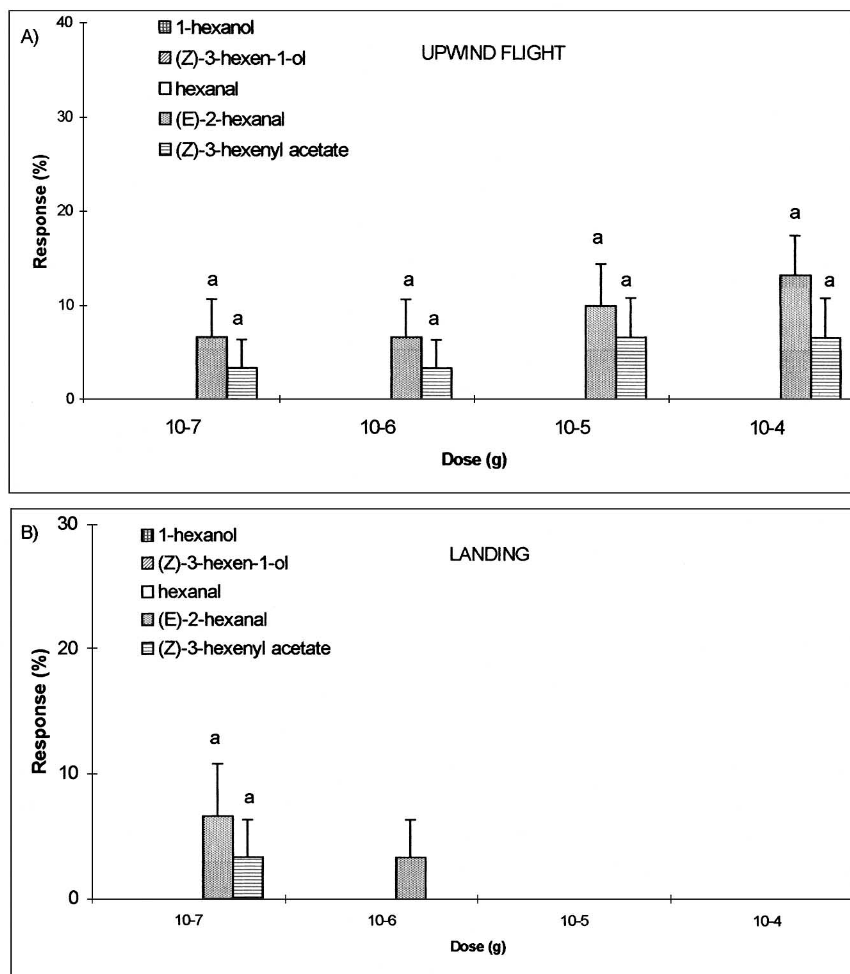

FIG. 5. Mean percentage (± SE) responses of mated females to green leaf volatiles at different doses: (A) upwind flight; (B) landing. Data are averages of six replications; in each, five insects were assayed. Bars marked by the same letter within doses are not significantly different ($P < 0.05$).

Chrysanthemum Volatiles

Active compounds identified in the chrysanthemum extract are shown in Table 2. The electrophysiological activity was confirmed by EAG recordings at the 10^{-5} g level by using authentic compounds (Table 2).

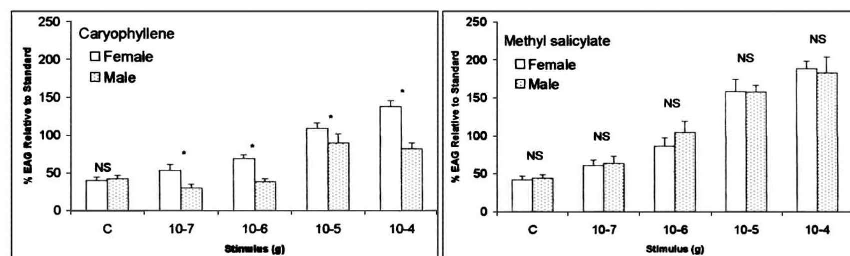

FIG. 6. Mean (± SE) EAGs of female and male *M. brassicae* to different doses of caryophyllene and methyl salicylate. C = control; NS = no significant sexual difference; *significant sexual difference, $P < 0.05$, t test assuming equal variances.

The electrophysiologically active compounds (except pinocarvone) in the chrysanthemum extract were evaluated individually in the wind tunnel at the 10^{-4} g level, except chrysanthenone, which was assayed at 10^{-5} g. In addition, the individual compounds that showed activity were evaluated in mixtures. Finally, all compounds (except pinocarvone) were blended in the relative proportions present in the extract, and the blend was evaluated. The results show that (*Z*)-3-hexenyl acetate, α-terpinene, 1,8-cineole, chrysanthenone, and camphor elicited upwind flight in mated females; there were no differences among these compounds ($F = 0.29$, $P = 0.881$). Only a few landings on targets were observed (Figure 7A).

TABLE 2. ANTENNAL EAG RESPONSES OF *M. brassicae* TO BIOLOGICALLY ACTIVE CHRYSANTHEMUM VOLATILES, EXPRESSED AS PERCENTAGE OF RESPONSE TO LINALOOL

Compound	EAG response to 10^{-5}-g dose (mean ± SE)	
	Male antenna	Female antenna
(*Z*)-3-Hexenyl acetate	75.2 ± 2.2**[a]	84 ± 5.3**
α-Terpinene	90.4 ± 14.9**	98.4 ± 11.5**
1,8-Cineole	95.4 ± 5.8***	90.8 ± 21.4*
α-Terpinolene	105 ± 11.5***	132 ± 15.4***
Linalool	100	100
Chrysanthenone	91.8 ± 11.4**	129.2 ± 12***
Camphor	126.6 ± 18.3***	153 ± 13.3***
Verbenol	122.4 ± 9.8***	92.6 ± 11.3**
Borneol	115.4 ± 13.2***	108.2 ± 3.7***

[a]*, **, ***: significantly different from control at $P < 0.05$, 0.01, and 0.001, respectively (t test assuming equal variances) (control = hexane, 10 μl).

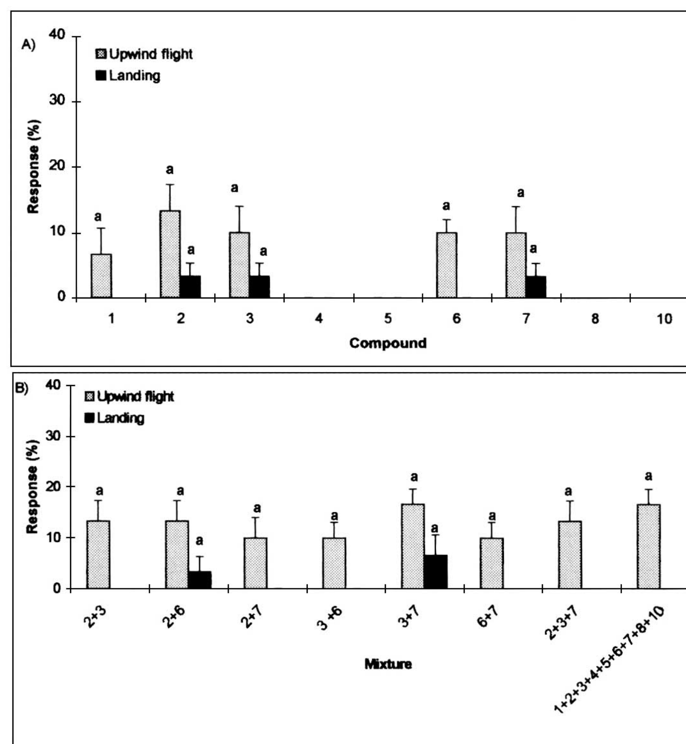

FIG. 7. Mean percentage (± SE) responses of mated females to electrophysiologically active chrysanthemum compounds evaluated individually (A) or in mixtures (B). Data are averages of six replications; in each, five insects were assayed. 1, (*Z*)-3-hexenyl acetate; 2, α-terpinene; 3, 1,8-cineole; 4, α-terpinolene; 5, linalool; 6, chrysanthenone; 7, camphor; 8, verbenol; 10, borneol.

There was no difference between the mixtures evaluated, including the nine-compound blend ($F = 0.31$, $P = 0.994$) (Figure 7B).

DISCUSSION

Electroantennogram and Behavioral Responses to Selected Brassicae Volatiles

Allyl NCS was an effective compound in stimulating upwind flight and landing of *M. brassicae* females, despite the lack of an EAG response by female antennae at the doses tested. In contrast, female antennae showed a stronger response to 4-pentenyl NCS, which was a less effective compound in the wind

tunnel. EAG does not always predict behavioral responses of insects to stimulating chemicals. For example, Pivnick et al. (1994) working with *Plutella xylostella* (L.), found that *n*-propyl NCS was not stimulatory to female antennae in EAG trials, but the females were attracted to this compound in field-trapping experiments. In other crucifer-feeding insects, a correlation between electrophysiology and behavior has been found. In the cabbage seed weevil, *Ceutorhynchus assimilis* Payk., 3-butenyl and 4-pentenyl NCS were attractive, while 2-phenylethyl NCS was not (Bartlet et al., 1993). A smaller EAG response to 2-phenylethyl than to 3-butenyl and 4-pentenyl NCS also has been found (Evans and Allen-Williams, 1992).

The fact that most NCS evaluated did not elicit an EAG response does not necessarily indicate the complete lack of olfactory cells, but rather may indicate the presence of a relatively small number of specialized olfactory cells. For *C. assimilis*, 3-butenyl and 4-pentenyl NCS, 1-8-Cineole, and (Z)-3-hexenyl acetate did not evoke significant EAG responses, but specific olfactory cells were discovered for these compounds by using single-cell recording techniques (Blight et al., 1995).

The response of the cabbage moth, a polyphagous insect, to allyl NCS is intriguing because this compound, as well as other isothiocyanates, is produced only in five dicotyledonous families, including the Brassicaceae (Fenwick et al., 1983). Cruciferous species are the preferred host plants of *M. brassicae* (Bretherton et al., 1979), and the insect may have evolved the ability to recognize a characteristic chemical of this family group. Isothiocyanates have been implicated as chemical cues mediating host-finding behavior of crucifer-feeding specialist insects and their natural enemies (Pivnick et al., 1994 and references therein). In contrast, the northern false chinch bug, *Nysius niger* Baker, a generalist insect, is strongly attracted to ethyl NCS, while allyl NCS and *n*-propyl NCS are less attractive (Pivnick et al., 1991).

The fact that allyl NCS evoked the strongest behavioral responses in *M. brassicae* females could be because it is the most prominent isothiocyanate in several cruciferous plants (Wallbank and Wheatley, 1976; MacLeod and Nussbaum, 1977; MacLeod and Pikk, 1978; Tollsten and Bergström, 1988). The effectiveness of the other isothiocyanates might be increased by using different doses from those evaluated here, although, higher doses also could be repellent. In *P. xylostella*, it has been reported that doses of 10^{-4} and 10^{-3} g of 3-methylpropyl and 2-phenylethyl NCS are repellent to females (Pivnick et al., 1994).

In this study, I found that only mated females flew upwind to allyl NCS, not virgin females or males. This suggests that it is used by mated females for finding plants for oviposition. Similar results have been found in *N. niger*, where mated females were predominantly attracted to ethyl NCS (Pivnick et al., 1991).

Isothiocyanates are released when plant tissue is damaged (Kjaer, 1976).

This suggests that allyl NCS is not the chemical responsible for eliciting upwind flight and landing of *M. brassicae* on undamaged plants (Rojas-León, 1997). Pivnick et al. (1994) found that allyl NCS, which was attractive to *P. xylostella*, was present only in homogenized plant volatiles and was practically lacking from intact plant volatiles. They found that a terpene-containing fraction in intact cabbage volatiles was attractive to the moths, but they were unable to isolate individual attractive compounds.

All the GLVs evaluated elicited significant EAG responses at doses of 10^{-6}, 10^{-5}, and 10^{-4} g. However, only (*E*)-2-hexenal and (*Z*)-3-hexenyl acetate stimulated upwind flight. The other GLVs evaluated are classified as neutral, since no effect on behavior was observed. Thibout et al. (1982) found (*Z*)-3-hexen-1-ol was attractive to *Acrolepiopis assectella* Zeller females, but 1-hexanol, (*E*)-2-hexen-1-ol, and (*E*)-2-hexenal showed no effect. Evans and Allen-Williams (1989) found that 1-hexanol was a strong repellent to both male and female seed weevils, *C. assimilis*.

That (*E*)-2-hexenal was found to elicit upwind flight of *M. brassicae* females is not surprising since this compound is released when cabbage plants are damaged (Wallbank and Wheathey, 1976; Tollsten and Bergström, 1988; Angelopoulos and Keller, 1994), and previous results with *M. brassicae* have shown that females prefer damaged cabbage plants to undamaged ones when compared in two-choice tests (Rojas, 1999b). Thus, this compound in combination with allyl NCS could be responsible for the preference.

Caryophyllene and methyl salicylate evoked significant EAG responses at doses of 10^{-6}–10^{-4} g in both male and female antennae. Despite this response, neither affected female behavior when evaluated in the wind tunnel. In other insect species, methyl salicylate has been shown to have repellent properties and to be associated with secondary metabolite-based defense in plants (Hardie et al., 1994; Pettersson et al., 1994; Losel et al., 1997). With other species it has been reported as an attractant, for example, Hammack (1997) found that it showed moderate attractiveness to western corn rootworm females, *Diabrotica virgifera virgifera* Leconte.

Chrysanthemum Volatiles

Of the compounds identified in the chrysanthemum extract, five influenced female behavior in the wind tunnel, and their effectiveness was not increased when they were combined. Many of the compounds identified in chrysanthemum extract are attractive to several other phytophagous species. For example, a mixture of plant volatiles containing (Z)-3-hexenyl acetate has been reported to attract *Leptinotarsa decemlineata* Say (Visser, 1986) and the scarab beetle, *Anomala octiescostata* Burmeister (Leal et al., 1994). From volatiles of banana cultivars susceptible to the banana weevil, *Cosmopolites sordidus* Germar, 1,8-

cineole was identified as one of the electrophysiologically active components (Ndiege et al., 1996).

The response of *M. brassicae* females to the volatiles identified in the chrysanthemum extract may possibly be increased by adding pinocarvone, a compound identified, but not evaluated. This compound could be the synergist lacking in the blend. Phelan et al. (1991) found that *Amyelois transitella* Walker females flew upwind and landed on a blend of five fatty acids; however, the attraction was only observed when oleic acid was present. In *H. armigera*, at least some of the six compounds have a synergistic effect, because removal of α-bulsene or α-humulene resulted in loss of attractivity of the blend (Hartlieb and Rembold, 1996).

It is interesting that some of the compounds identified in the chrysanthemum extract have been reported previously in other host plants of *M. brassicae*. 1,8-Cineole and (Z)-3-hexenyl acetate are emitted by intact cabbage plants (Tollsten and Bergström, 1988; Blaakmeer et al., 1994; Angelopoulos and Keller, 1994). Omata et al. (1990), found (*Z*)-3-hexenyl acetate and camphor in the volatiles of apple. α-Terpinene and 1,8-cineole have been reported in tomato leaves (Buttery et al., 1987). Nevertheless, a complex of volatiles rather than one single compound is fundamental for the attraction of the phytophagous insects (Visser, 1986).

Of the compounds identified in the chrysanthemum extract that elicited upwind flight of *M. brassicae* females, only three stimulated landing on targets. This suggests that other compounds, in addition, may be responsible for eliciting landing. Hartlieb and Rembold (1996) found that α-humelene and α-bulsene elicit different behavioral responses in *H. armigera*. The latter compound principally stimulated approaches to the targets, whereas α-humelene influenced the landing rate. Oleic acid was found to elicit upwind flights of *A. transitella* females to the source; however, short-range responses were enhanced by the addition of linoleic acid, which elicited no long-range orientation (Phelan et al., 1991).

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MOSQUITOCIDAL ACTIVITY OF ACETYLENIC COMPOUNDS FROM *Cryptotaenia canadensis*

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Abstract—As part of an on-going program to identify natural products from plants with mosquitocidal activity, two acetylenic compounds were isolated from *Cryptotaenia canadensis*, a native North American umbellifer frequently encountered in moist woodlands. Fresh foliage, roots, and fruits were extracted with a hot methanol and water mixture and then dried. The extract was partitioned into chloroform and water, and both phases were bioassayed against fourth instars of *Culex pipiens* at concentrations between 5 and 50 ppm. Only the organic phase was active. Gas-liquid chromatography revealed that the two main components of the organic phase were polyacetylenes. The compounds were isolated by vacuum chromatography on silica gel and identified as falcarinol and falcarindiol based on GC-MS-EI and GC-MS-CI fragmentation patterns, high-resolution MS, and ^{1}H and ^{13}C NMR spectrometric data. The dose–response curves with mosquito larvae were determined by probit analysis. The LC_{50} values were 3.5 ppm for falcarinol and 6.5 ppm for falcarindiol. The distribution of polyacetylenes varied among plant parts. Fruits contained an unknown compound not found in either the foliage or roots.

Key Words—Apiaceae, Insecta, mosquito, umbellifer, polyacetylene, falcarinol, falcarindiol, larvicide.

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INTRODUCTION

Plants synthesize a variety of chemically diverse secondary metabolites, at least in part in response to selection pressures from herbivores and microorganisms (Harborne, 1971). The biological activity of these natural products can be exploited by screening plant extracts against nonadapted organisms or cell and tissue cultures. Such studies have resulted in the discovery of numerous phytoalexins, insecticides, nematocides, antibiotics, and anticarcinogenic compounds (Lewis, 1977; Robinson, 1991; Frohne and Jensen, 1992). Over the past three years, we have evaluated the mosquitocidal activity of several hundred native Illinoisian plants. One of the most toxic extracts to *Culex pipiens* larvae was from *Cryptotaenia canadensis* (L.) DC. (Apiaceae), a moist woodland umbellifer common in the United States east of the Rocky Mountains (Jones and Fuller, 1955).

Within the Apiaceae, there are four major groups of secondary plant compounds: terpenes, phenylpropanoids, furanocoumarins, and polyacetylenes. The characteristic volatile terpenes and phenylpropanoids of some umbellifers are the basis for their use as spices and medicinal drugs (Frohne and Jensen, 1992). Linear furanocoumarins are known for their ability to cross-link strands of DNA in the presence of UV light (Camm et al., 1976; Berenbaum, 1978). Polyacetylenes, particularly long-chain acetylenic alcohols and ketones, exhibit varied pharmacological effects (Robinson, 1991).

Polyacetylenes are not widely distributed among plant families; they are found mainly in the Apiaceae, Araliaceae, and Asteraceae. Their limited distribution, together with other chemical and morphological characteristics, led to the postulation that the Araliaceae and Apiaceae belong to the same order (Araliales) and that the Asterales evolved from the Araliales (Hegnauer, 1971; Holub et al., 1987). Bohlmann et al. (1973) reported two diacetylenes, a C_{17}-monoalcohol and a C_{17}-ketone, in the roots of *C. canadensis* and traces of a monoacetylene in the leaves. There are no additional reports of secondary metabolites from *C. canadensis* or of biological activity of extracts from this plant.

The purpose of this investigation was to identify and isolate the major components of mosquitocially active extracts of *C. canadensis* and to determine the relative larvicidal activity of the purified chemicals.

METHODS AND MATERIALS

Plant Extracts. Fresh plant material was extracted for ca. 20 min with boiling methanol–water 80 : 20) and then filtered once through cheesecloth and twice through Whatman filter paper No. 4. The methanol was removed under vacuum, and the remaining extract was partitioned into aqueous and chloroform phases.

Both phases of the crude extract were examined for mosquitocidal activity. The active chloroform phase was further fractionated by vacuum chromatography on a silica gel column with a petroleum ether–chloroform–methanol gradient. The fractions were examined on silica gel TLC plates with a 254-nm fluorescence indicator and the acetylenes were visualized under UVB light. The mobile phase was chloroform–methanol (95 : 5).

The fractions that contained acetylenes were treated with activated charcoal to remove chlorophyll. The purity of each fraction was ascertained by gas chromatography (GC). The mass was determined with GC-MS-CI (methane) and the molecular formula was determined with high resolution MS-CI. ^{1}H NMR and ^{13}C NMR data were recorded at 500 MHz, with the solvent $CDCl_3$ as reference (7.26 ppm, ^{1}H NMR; (77.0 ppm, ^{13}C NMR).

The leaves, fruits, and roots were extracted separately three times in order to determine whether the plant parts varied in acetylenic content. The percentage of water in the different parts of the plants was estimated by comparing the weight of freshly collected material to its weight after drying at 26°C for five to seven days (when weight loss stabilized).

Bioassays. Initial screening of larvicidal activity used whole plant extracts of *C. canadensis*. The dried chloroform phase was resuspended in acetone at 10 mg/ml (10,000 ppm) and the aqueous phase in water at the same concentration. The isolated diacetylenes were each brought to a concentration of 2 mg/ml in acetone (2000 ppm). The solutions were tested in a 600-ml beaker containing 200 ml deionized water and 20 fourth instars of *Culex pipiens* (Diptera: Culicidae). The crude extract was bioassayed at concentrations ranging from 5 to 50 ppm and the pure compounds were assayed at five concentrations between 1 and 10 ppm. This procedure was repeated five times. Controls consisted of 20 fourth instars in deionized water.

For the evaluation of phototoxicity, third instars, treated as described above, were exposed to predominantly UVA light (430 $\mu W/cm^2$) for 2 hr. A control set of larvae was placed on the shelf underneath and shielded from the UV light.

Mortality was measured as the number of dead and moribund larvae after 24 and 48 hr. The LC_{50}, LC_{95}, and LC_{99} were calculated by probit analyses (SPSS for Windows, SPSS Inc., Chicago, Illinois).

RESULTS AND DISCUSSION

The chloroform phase from the methanol extract of *C. canadensis* was highly active against *Culex pipiens* larvae in our assay. The LC_{50} of the whole plant extract at 24 hr was 14 ppm. GC analysis revealed two major peaks in a ratio of 3.8 : 1 in the whole plant (Figure 1). A CI mass spectrum of the larger peak gave a molecular weight of 260 and high-resolution MS suggested the

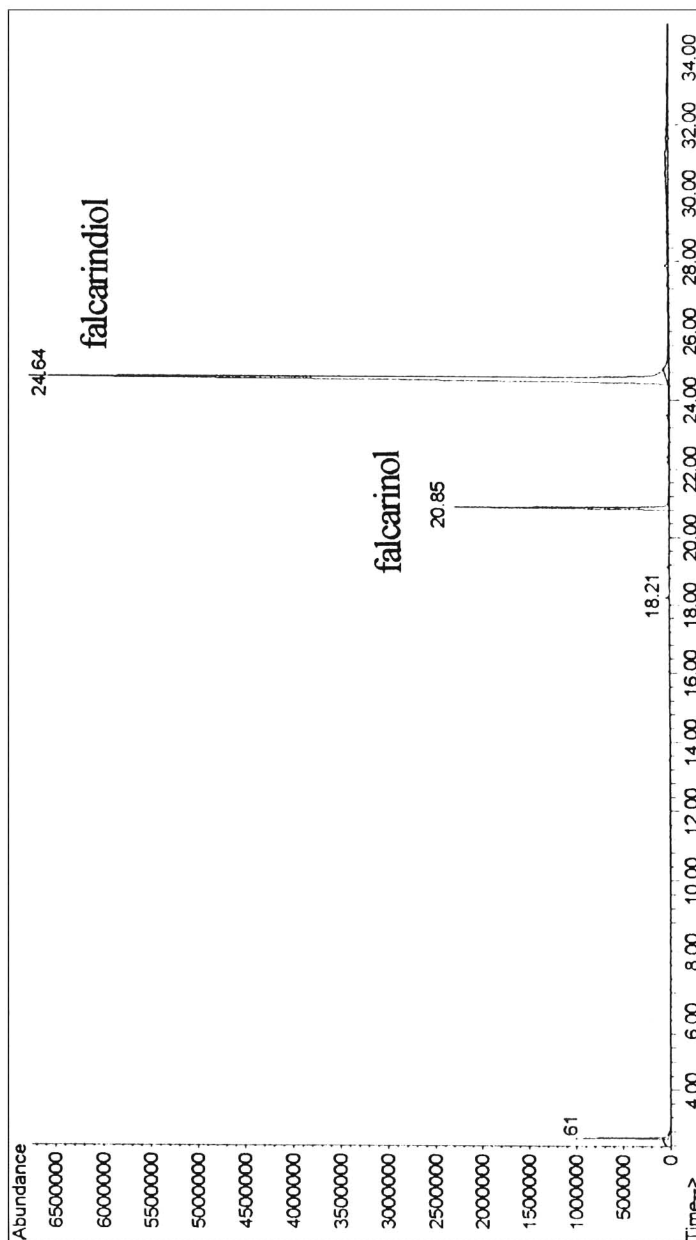

FIG. 1. Gas chromatogram of the chloroform phase of *Cryptotaenia canadensis*.

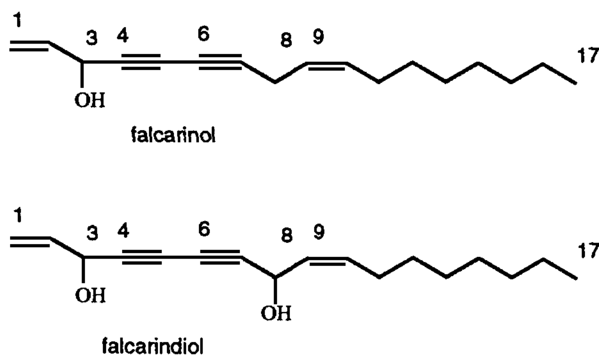

FIG. 2. Structures of the diacetylenes falcarinol and falcarindiol.

molecular formula of $C_{17}H_{24}O_2$. ^1H NMR revealed the following peaks: δ 0.88 (3H, t, J = 13.5 Hz, 17-Me), δ 1.27 (8H, m, 13-CH_2 to 16-CH_2), δ 1.38 (2H, m, 12-CH_2), δ 2.11 (2H, two dd, J_1 = 7.1 Hz, J_2 = 1.2 Hz, 11-CH_2), δ 4.95 (1H, d, J = 5.2 Hz, H-3), δ 5.20 (1H, d, J = 8.4 Hz, H-8), δ 5.26 (1H, d, J_1 = 10.2 Hz, H-1b), δ 5.46 (1H, d, J_1 = 17.0 Hz, H-1a), δ 5.52 (1H, dd, J_1 = 10.0 Hz, J_2 = 8.4 Hz, H-9), δ 5.61 (1H, two dd, J_1 = 10.0 Hz, J_2 = 7.1 Hz, H-10), δ 5.94 (1H, ddd, J_1 = 17.0 Hz, J_2 = 10.2 Hz, J_3 = 5.2 Hz, H-2). The ^{13}C NMR spectra had absorptions at: δ 14.1 (C-17), δ 22.6 (C-16), δ 27.7 (C-11), δ 29.1 (C-12, C-13), δ 29.2 (C-14), δ 31.8 (C-15), δ 58.6 (C-8), δ 63.5 (C-3), δ 68.7 (C-6), δ 70.3 (C-5), δ 78.2 (C-4), δ 79.8 (C-7), δ 117.4 (C-1), δ 127.6 (C-9), δ 134.7 (C-10), and δ 135.8 (C-2). Based on these data, the purified compound was identified as falcarindiol [heptadeca-1,9(*cis*)-diene-4,6-diyne-3,8-ol] (Figure 2). The NMR spectra correspond to those previously published (Kern and Cardellina, 1982; Miyazawa et al., 1996). The smaller GC peak was identified as falcarinol [heptadeca-1,9(*cis*)-diene-4,6-diyne-3-ol] (Figure 2) with a molecular weight of 244 and molecular formula of $C_{17}H_{24}O$. The NMR data were essentially identical to those previously reported (Gafner et al., 1989; Hansen and Boll, 1986; Bohlmann et al., 1966). Falcarinol was eluted from a vacuum column with 2–10% methanol in chloroform and falcarindiol was eluted with 15–30% methanol in chloroform.

The diacetylenes falcarinol and falcarindiol have previously been isolated from other species of the Apiaceae and Araliaceae (Bohlman et al., 1973; Kern and Cardellina, 1982; Yates and England, 1982; Miyazawa et al., 1996; Villegas et al., 1987; Bruhn et al., 1987). Falcarinol can be a potent allergen (Bruhn et al., 1987; Hansen and Boll, 1986) and is highly cytotoxic (Bernart et al., 1996). Falcarindiol, in contrast, has recognized antifungal, antimutagenic, and antiproliferative properties (Davies and Lewis, 1981; Garrod et al., 1978; Kemp, 1978;

Ikeda et al., 1998). Mosquitocidal activity from these polyacetylenes has not been reported previously.

Bohlmann et al. (1973) correctly reported falcarinol from the roots of *C. canadensis;* however, he also reported a C_{17}-ketone from the roots and traces of a monoacetylene in the leaves, both of which we were unable to confirm. This is the first time that falcarindiol has been identified as a major constituent of *C. canadensis*. The concentration of falcarindiol in the whole plant is 174.4 $\mu g/g$ fresh weight and that of falcarinol 46.5 $\mu g/g$. In the roots, falcarindiol makes up more than 95% of the acetylenes present in the chloroform phase of the methanol extract. The concentration of falcarindiol in the roots was 68.2 $\pm$ 3.2 $\mu g/g$ fresh weight (N = 3). In the fruits, falcarindiol is also a dominant acetylene. It occurred at 1.2 mg/g $\pm$ 3.7 $\mu g/g$ fresh weight (N = 3) and was about 94% of the total acetylenes present in the fruits. Thus, in both fruits and roots, the diol is the dominant acetylenic component. In contrast, the average concentration of falcarinol in the leaves is slightly higher than the concentration of falcarindiol. Falcarinol occurs at 220.4 $\pm$ 7.1 $\mu g/g$ and falcarindiol at 146.9 $\pm$ 6.9 $\mu g/g$ fresh weight in leaves.

The concentration of acetylenes by fresh weight was significantly greater in fruits than leaves. However, if one corrects for water content of leaves (about 75%) and fruits (about 3%) then the total amount of acetylenes based on dry weight was in the same range (about 1.3 mg/g dry weight of fruits and 1.5 mg/g dry weight of leaves).

With a water content of about 55% in the roots, the amount of falcarindiol was, based on the dry weight (151 $\mu g/g$), much lower than in leaves (881.6 $\mu g/g$) and fruits (1207.0 $\mu g/g$). It is interesting that the amount of falcarindiol in the roots was lower than in the aerial parts. If its main biological activity is antifungal (Davies and Lewis, 1981; Garrod et al., 1978), then the highest concentration would be expected in the roots. Considering the insecticidal activity of falcarindiol to mosquito larvae, it is possible this polyacetylene may serve as a protectant against herbivory.

Fruit extracts did have a distinct component that was not found in either leaves or roots. This compound was eluted from the vacuum column in the same fractions as falcarinol (2–10% methanol in chloroform), GC-MS analysis (Figure 3) suggests a probable parent ion for this unidentified compound at m/z = 218.

The isolated acetylenic compounds were very active against *Culex pipiens* larvae. The LC_{50} for falcarinol was 3.5 ppm (24 hr) and 2.9 ppm (48 hr), and the LC_{50} for falcarindiol was 6.5 ppm (24 hr) and 4.5 ppm (48 hr) (Table 1). Falcarinol, the more lipophilic compound, was more toxic than falcarindiol, the more polar acetylene.

Insecticidal activity from apiaceous diacetylenes has not been previously reported, although some of the more highly oxidized polyacetylenes from Asteraceae are toxic to mosquito and blackfly larvae, especially under sunlight or UV

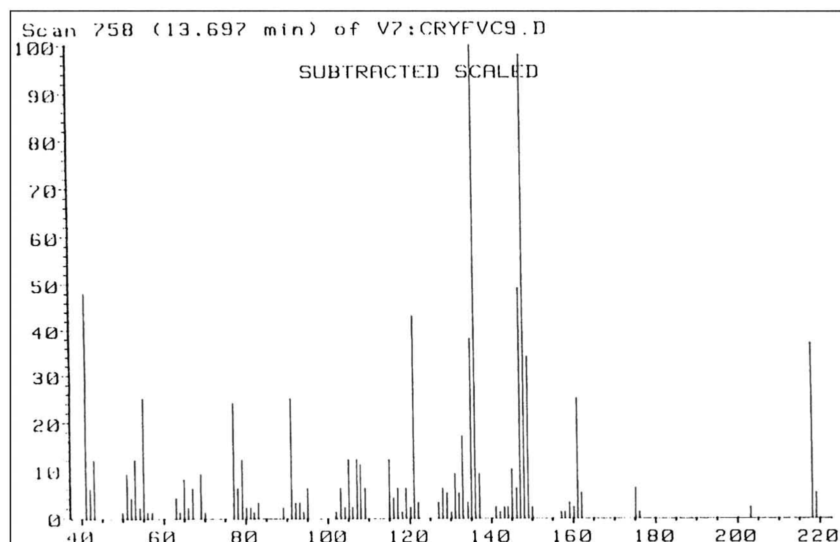

FIG. 3. Mass spectrum of an unknown compound from the fruits of *C. canadensis*.

light (Wat et al., 1981; Arnason et al., 1981). Our investigation with falcarindiol showed no photoactivation with UVA radiation. For third-instar *C. pipiens*, the LC_{50} after 24 hr was 3.9 ppm for the control and 4.2 ppm for larvae exposed to predominantly UVA light (430 $\mu W/cm^2$) for 2 hr ($N = 3$). Under these conditions, we found no abnormally high mortality among the untreated controls

TABLE 1. LARVICIDAL ACTIVITY OF FALCARINOL AND FALCARINDIOL AGAINST *Culex pipiens*

	Lethal concentrations (ppm) and 95% confidence intervals			
Compounds	LC_{50}	LC_{95}	LC_{99}	Slope
Falcarinol				
24 hr	3.49	11.93	19.85	3.08
	(3.04–4.15)	(8.45–12.04)	(12.59–45.19)	
48 hr	2.91	9.48	15.47	3.21
	(2.53–3.35)	(6.99–16.55)	(10.23–33.41)	
Falcarindiol				
24 hr	6.51	24.24	42.23	2.87
	(5.54–8.31)	(16.46–45.88)	(25.70–93.66)	
48 hr	4.51	17.01	29.50	2.85
	(3.84–5.56)	(11.81–30.44)	(18.50–65.51)	

exposed to the UV light. Both LC_{50} values were lower than those reported above for fourth instars, suggesting toxicity may decrease with development. Younger larvae may be more sensitive to these acetylenes because of smaller body size.

The mode of action of falcarinol and falcarindiol on mosquito larvae was not investigated; however, the mechanism for antifungal activity of falcarindiol is believed to involve disruption of cell membranes (Garrod et al., 1979). Falcarinol may be more toxic to mosquito larvae than falcarindiol due to its lipophilic nature, thus providing a greater penetration of the cuticle and cell membranes.

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CHEMICAL AND CHROMATIC BASES FOR PREFERENTIAL VISITING BY THE CABBAGE BUTTERFLY, *Pieris rapae*, TO RAPE FLOWERS

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Abstract—Scent and coloration of corolla were examined as floral attributes responsible for preferential visiting by the cabbage butterfly, *Pieris rapae*, to rape flower, *Brassica rapa*. Floral volatile components that release the flower-visiting behavior of the butterfly were identified by chemical analyses, electroantennography (EAG), and two behavioral bioassays: proboscis extension reflex (PER) in response to odor and attraction to artificial flowers. GC and GC-MS analyses of the headspace volatiles from the flowers revealed the presence of six aromatic compounds, benzaldehyde, phenylacetaldehyde, benzyl alcohol, 2-phenylethanol, phenylacetonitrile, and indole in decreasing order of quantity. Of these, phenylacetaldehyde elicited the highest response in the PER assay. While benzyl alcohol, 2-phenylethanol, benzaldehyde, and phenylacetonitrile evoked moderate responses, the PER-eliciting activity of indole was very weak. In two-choice behavioral bioassays, artificial flowers scented with any one of these PER-active compounds attracted significantly more butterflies than control (unscented) flowers, whereas those treated with indole were almost inactive. The EAG activities of the six chemicals were not high and were about the same at a low dose (1 μg), but phenylacetaldehyde elicited a much stronger response from both sexes at higher doses (10 and 100 μg). An overall profile of EAG responses at a dose of 100 μg was analogous to that of PER performance, suggesting that floral volatiles may be involved in close-range location or recognition of flowers rather than long-range attraction. By spectroscopic and UV-photographic examinations of rape flower, the central part of the corolla was found to absorb UV rays in marked contrast to the other parts, which reflected near-UV rays (λ_{max} = 350 nm). This indicates that the flower is endowed with a conspicuous nectar guide that is probably an important visual stimulus for attracting foraging adults of *P. rapae*. Consequently, the present findings strongly suggest that this elaborate pollination strategy of rape flower, characterized by its good combination of

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olfactory and visual attractiveness, accounts for preferential visiting by the cabbage butterfly to the flower.

Key Words—Flower-volatiles, floral scent, *Pieris rapae*, *Brassica rapa*, proboscis extension reflex, EAG, benzaldehyde, phenylacetaldehyde, 2-phenylethanol, phenylacetonitrile, UV nectar guide.

INTRODUCTION

Angiosperms have evolved unique floral characteristics such as shape, color, scent, nectar production, and flowering season mainly to attract pollinators. Butterflies visit flowers primarily to obtain energy in the form of nectar, and simultaneously they serve as pollinators (Dobson, 1994). Most butterfly species, however, do not forage on all flowers available, but often consistently visit specific flowers (Lewis, 1989; Weiss, 1995; Kandori and Ohsaki, 1996; Goulson et al., 1997). For a comprehensive understanding of the evolutionary associations between flowers and butterflies, it is essential to elucidate which floral features are involved in their foraging behavior.

Pieris rapae crucivora (Pieridae) is thought to be an important pollinator for many plant species, since the butterfly occurs three to four times a year and tends to visit a diversity of flowers. It has been reported that the butterfly primarily utilizes color vision for flower selection and prefers purple, blue, and yellow flowers (Kay, 1976; Miyakawa, 1976; Kandori and Ohsaki, 1996). Nectar guides are also known to act as a signal for attracting foraging adults and to promote proboscis extension (Kandori and Ohsaki, 1996, 1998). Despite such strong reliance on colors, *P. rapae* also makes use of olfactory cues from flowers during foraging; our previous study has shown that certain floral volatiles of *Ligustrum japonicum* (Oleaceae), such as 2-phenylethanol, phenylacetaldehyde, 6-methylhept-5-en-2-one, benzaldehyde, and methyl phenylacetate significantly stimulate foraging by *P. rapae* (Honda et al., 1998).

In Japan, *P. rapae* frequently visits rape flowers, *Brassica rapa* (Cruciferae), in early spring. Although the bright yellow coloration of the petal seems highly attractive to the butterfly (Miyakawa, 1976), it apparently prefers this flower to others of similar coloration blooming in the same season. We thus supposed that floral characteristics other than visible coloration might add to the attractiveness of the flower. The present work deals with the chemistry of scent components of rape flower, their effects on foraging by *P. rapae*, and a chromatic analysis of corolla of the flower.

METHODS AND MATERIALS

Insects. Adults of *P. rapae* (2–7 days old) used for the experiments were obtained from our laboratory stock cultures. Larvae were reared on potted cab-

bage at 25°C under a 16L : 8D photoperiod. Adults were fed with 10% sucrose water solution once daily and were not allowed to contact real flowers throughout the experiments. All behavioral and electrophysiological experiments were conducted at 25°C in an air-conditioned room with good ventilation.

Collection of Headspace Volatiles from Flowers. A total of 120 intact flowers of potted *B. rapa* were covered with a cylindrical plastic vessel (25 cm ID × 40 cm) equipped with a Teflon tube air outlet. Air in the vessel was purged by an air pump at a rate of 2.0 liters/min through the outlet, which was connected to a glass column (15 mm ID × 80 mm) packed with 1 g of Tenax-GR, while admitting fresh air into the vessel from the open end. Headspace volatiles were continuously collected for 10 hr. Volatiles adsorbed on Tenax were extracted with 30 ml of isopentane for 12 hr at room temperature and then concentrated to 100 μl in vacuo at 15°C. The sampling was repeated three times, each time with 120 flowers. Miscellaneous impurities present in air and the solvent were checked by GC and GC-MS.

Solvent Extraction of Flower. To examine the actual quantities of volatile components contained in flowers, 120 excised flowers (the average fresh weight was 31.4 mg per flower) were extracted immediately after collection with 80 ml of isopentane for 30 min at 15°C. The extract was concentrated to 100 μl under a nitrogen stream at 0°C. The sampling was replicated twice.

Chemical Analysis of Floral Volatiles. The chemical compositions of both headspace and solvent-extracted samples were analyzed by GC and GC-MS. GC analyses were conducted with a Shimadzu GC-14A gas chromatograph on an FFAP fused-silica capillary column (0.25 mm ID × 50 m). The injection temperature was 250°C, and the oven temperature was programmed from 50°C to 220°C at a rate of 5°C/min. The flow rate of carrier gas (N_2) was 1 ml/min. EI-MS spectra were recorded at an ionization potential of 70 eV on a Shimadzu QP-2000 mass spectrometer connected to the same GC model with the same capillary column and under similar operating conditions as above. CI-MS and HR-MS spectra were recorded on a Jeol JMS-DX303 mass spectrometer coupled with a Hewlett Packard HP5890 gas chromatograph containing an Ultra Alloy-FFAP capillary column (Frontier Lab., 0.25 mm ID × 30 m) and with similar operating conditions as above. Identification of components was based on comparisons of GC retention data and mass spectra with those of authentic samples, unless otherwise noted. A rough estimate of the quantities of individual components was made on the basis of their GC peak areas in terms of 2-phenylethanol.

Proboscis Extension Reflex (PER). Since most butterfly species usually extend their proboscis in response to food odors (Honda, 1973, 1976; Honda et al., 1998), adult butterflies were tested for PER performance with six floral volatiles detected from the headspace sample according to the method of Honda et al. (1998). Prior to the experiments, butterflies were conditioned in the following manner: adults that had been starved for about 20 hr were permitted free

flight for 3 hr in a plastic chamber (30 × 25 × 20 cm) under incandescent lamps (3500 lux) and were subsequently fed only water. One individual was introduced into a plastic mesh cage (15 × 13 × 8 cm) under similar illumination. A test chemical (5 μl or 5 mg) was applied to a filter paper strip (5 × 30 mm), which was attached to the end of a glass tube (5 mm ID × 30 mm). The odor plume of the test chemical was delivered through a glass tube onto the antenna for 30 sec together with a carrier gas (deodorized and humidified air; 1400 ml/min) from outside of the cage. Each individual was assayed twice a day, and the tests were repeated for two consecutive days. The proboscis extension response to a test chemical was scored as 100 (%) for complete extension of the proboscis, 50 (%) for half-unrolling the proboscis and 0 for no response. The PER-eliciting activity of each compound was expressed as the mean percentage of responses recorded from 40 individuals of each sex. The significance of differences among the responses was assessed by a *t* test.

Tests for Attraction to Artificial Flowers. The effect of floral scent on a butterfly's alighting and on postalightment behavior (PER) was examined with scented and unscented flower models by a method similar to that of Honda et al. (1998). The model consisted of a 50-ml Erlenmeyer flask (75 mm high) laterally covered with green paper, a doughnut-shaped yellow filter paper disk (22 mm ID, 50 mm OD) held horizontally on the top of the flask, and a crumpled white paper towel inserted into the flask such that it protruded about 1 cm above the top. The flask was filled with distilled water. Samples tested were six floral components, and a flower-scent mimic (synthetic blend) that was a mixture of five compounds (benzyl alcohol, 2-phenylethanol, benzaldehyde, phenylacetaldehyde, and phenylacetonitrile) prepared to approximate the blend ratios of the components found in the solvent extract. When individual chemicals were tested, each chemical (5 mg) was applied to the paper towel (treated model), while the unscented model was used as the control. For the test with the synthetic blend, 25.3 μg of the sample (equivalent to the sum total of the volatiles from approximately 300 flowers) dissolved in 50 μl of dichloromethane was applied to the paper towels of treated models, while the control was treated with an equal amount of the solvent only. Two treated and two control flowers were placed diagonally across from each other at the corners of a square (50 × 50 cm) in the experimental arena (150 × 70 cm, 76 cm high, 3500 lux), the floor of which was covered with green plastic plates to imitate green vegetation. Before the bioassay, each individual was conditioned in the same manner as in the PER assays. Thirteen adults (sex ratio not controlled) were employed for each test and allowed free flight over the experimental arena. The number of alightings followed by PER during a 30-min period was recorded for the treated flowers and the controls. During the bioassay, the positions of the flowers were rotated once every 10 min. Tests for each chemical were replicated three times with different individuals. The significance of differences between treatments and controls

was analyzed by a Wilcoxon's signed-ranks test, while that between the synthetic blend and each chemical was assessed by a χ^2 test.

Electroantennogram (EAG) Recording. Antennal sensitivity to the six floral compounds was tested electroantennographically by a method similar to that reported previously (Honda et al., 1998). A test compound dissolved in dichloromethane was deposited on a filter paper strip (5 × 30 mm). After allowing the solvent to evaporate at room temperature, we inserted the filter paper into a glass tube (6 mm ID × 50 mm). An odor puff of 1 ml was delivered onto the antenna preparation together with a deodorized and humidified air stream (350 ml/min), which was constantly blowing over the antenna. Each compound was tested at three doses (1, 10, and 100 μg) on five different antennae of each sex. All tests were replicated three times for each preparation. Responses to a blank puff and a standard chemical (1 μg of hexan-1-ol) were recorded for every two measurements of each compound in order to rectify the time-lapse change of antennal sensitivity and the interindividual variation in absolute response. The responses were averaged and expressed as percentages of the response to the standard in each sex. The differences in EAG intensities were analyzed by a *t* test.

Test Chemicals. Authentic chemicals used as olfactory stimuli were commercially available (Tokyo Chemical Industry and Aldrich). Their purities, as assessed by GC, were all above 97%.

Reflection Spectra of Flower Petal. Reflection spectra of rape flower petals were measured with a Shimadzu UV-3100 UV-VIS-NIR scanning spectrophotometer. The petal excised from a flower was placed flat on a blank plastic plate (25 × 30 mm) with adhesive tape, and the preparation was fixed on a solid sample folder.

Ultraviolet Photography. UV photographs of the corolla of rape flowers (Fuji Neopan 400, ASA-400, $f = 4.0$–8.0, 1/4–1/15 sec) were taken with a (Pentax Z-1) camera with a 50-mm macro lens (Pentax 50 macro) through a visible-light-absorbing filter (Kodak Wratten 18A) (Horovitz and Cohen, 1972; Utech and Kawano, 1975) under two types of fluorescent lamps: National FL40SBA-37 and FL40SPG.

RESULTS

Chemical Composition of Floral Scent. Six aromatic compounds were identified from the headspace sample, of which the major components were benzaldehyde and phenylacetaldehyde (Table 1). In contrast, floral volatiles prepared by solvent extraction contained large amounts of nitriles and isothiocyanates (Kjær et al., 1963), as well as the above aromatic compounds. These nitrogenous components are considered to be damage-induced artifacts produced by the enzymatic degradation of glucosinolates (Cole, 1976).

TABLE 1. CHEMICAL COMPOSITION OF FLORAL VOLATILES OF *Brassica rapa* L.

Compound	HS sampling (N = 3) content [ng, (avg.)][a]	Solvent extraction (N = 2) content [ng, (avg.)][b]
Aliphatics		
(*Z*)-Hex-3-en-1-ol		10.32–30.37 (20.34)
Aromatics		
Benzaldehyde	2.98–22.17 (8.36)	22.79–30.37 (26.58)
Phenylacetaldehyde	1.92–7.92 (3.17)	6.15–11.40 (8.78)
Benzyl alcohol	0.08–2.23 (0.75)	2.97–3.31 (3.11)
2-Phenylethanol	0.31–1.89 (0.56)	7.74–13.50 (10.62)
Methyl 3,5-dimethoxybenzoate		11.23–17.36 (14.30)
Methyl 3,4,5-trimethoxybenzoate		11.23–35.68 (23.46)
Benzyl benzoate		132.03–186.16 (159.10)
Indole	trace–0.08 (0.05)	19.28–33.77 (26.53)
Nitriles		
Pent-4-enenitrile[c]		137.39–257.18 (197.29)
Hex-5-enenitrile[c]		303.41–678.70 (491.06)
Phenylacetonitrile	0.31–0.62 (0.46)	6.15–11.41 (8.78)
3-Methylthioallylnitrile[c]		86.93–137.98 (112.46)
3-Phenylpropionitrile		86.06–110.97 (98.52)
4-Methylthiobutanenitrile[c]		52.98–191.25 (122.12)
Isothiocyanates		
But-3-en-1-yl isothiocyanate		50.04–85.17 (67.61)
Pent-4-en-1-yl isothiocyanate[c]		145.97–172.08 (159.03)
2-Phenylethyl isothiocyanate		32.45–51.87 (42.16)

[a]Amount emitted per hour from one flower.
[b]Content per flower (average fresh weight: 31.4 mg per flower).
[c]Tentative identification from mass spectrum.

PER in Response to Floral Components. Although the PER performance of *P. rapae* was not remarkable on the whole, phenylacetaldehyde elicited the highest response from both sexes (ca. 30%), while 2-phenylethanol, benzyl alcohol, benzaldehyde, and phenylacetonitrile evoked moderate responses (ca. 20%) (Figure 1). In contrast, the responses to indole were lower than those to phenylacetaldehyde and 2-phenylethanol ($P < 0.01$) in both sexes.

Effect of Floral Odor on Flower-Visiting Behavior. It is apparent that certain floral volatiles notably enhanced the frequency of alighting accompanied with proboscis extension (Figure 2). The proportion of the number of alightings on treated flowers to that on control flowers calculated for each set is also shown in Figure 2. Benzaldehyde (6.22), phenylacetaldehyde (4.71), and 2-phenylethanol (4.70) were very effective in stimulating foraging ($P < 0.01$), while phenylacetonitrile (3.82), and benzyl alcohol (1.98) were moderately or slightly effective ($P < 0.05$). In contrast, indole (1.32) exerted neither a posi-

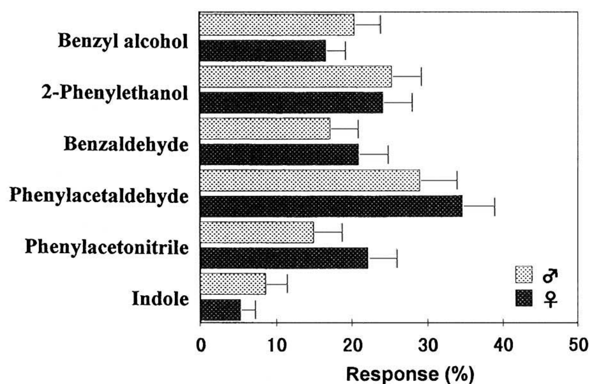

FIG. 1. PER performance (mean ± SE) of *P. rapae* adults ($N = 40$) in response to six floral components from *B. rapa*.

tive nor negative effect on foraging. Although the synthetic blend was tested at a much smaller dose (1/200) than single compounds, the blend attracted more butterflies (4.85, $P < 0.01$), with an attractiveness almost comparable to that of phenylacetaldehyde or 2-phenylethanol.

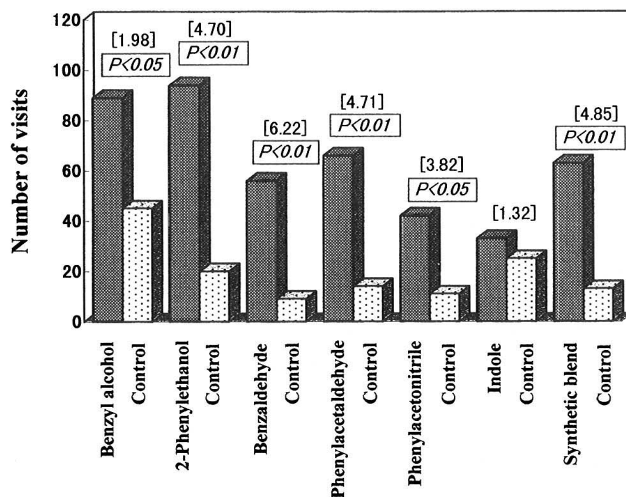

FIG. 2. Effect of floral scent components (5 mg each) and a synthetic blend (25.3 μg) on the attraction of *P. rapae* adults ($N = 39$) to artificial flowers. Alightings on treated flowers followed by PER in proportion to those occurring on control flowers is given in brackets.

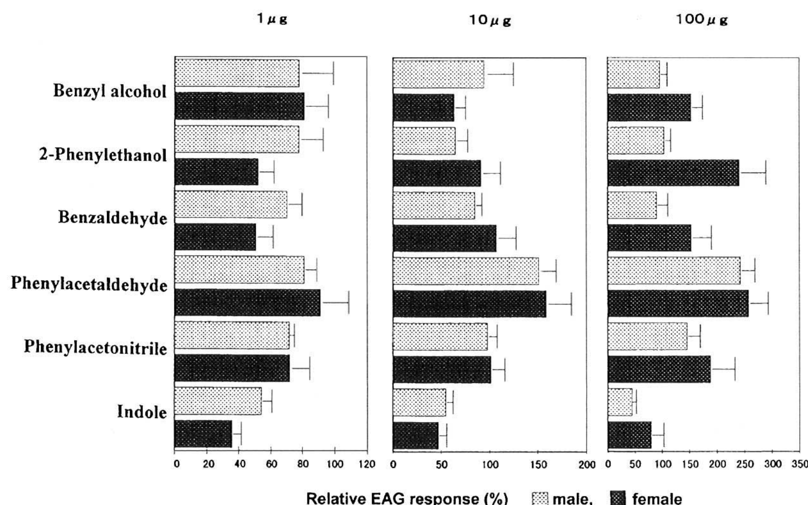

FIG. 3. Relative EAG responses (mean ± SE) of *P. rapae* adults ($N = 5$) to three doses of individual scent components.

EAG Responses to Floral Components. Relative EAG responses (mean ± SE) of both sexes to the six floral volatiles are shown in Figure 3. At a dose of 1 μg, EAG intensities of these compounds were not greatly different from one another, although indole elicited a slightly weaker response. At higher doses (10 and 100 μg), phenylacetaldehyde released the highest response from both sexes, while indole remained least active. The difference in intensities between the two compounds was significant ($P < 0.01$). Sexual difference was not significant at doses smaller than 10 μg. At 100 μg, however, females responded more strongly to 2-phenylethanol than did males ($P < 0.05$), and the female responses to benzyl alcohol and benzaldehyde was also slightly higher than the male responses.

Characteristics of Flower Coloration. The petals of *B. rapa* flower are uniformly bright yellow in color. The outer edge showed a characteristic reflection spectrum in the range below 400 nm that was considerably different from that of the central part; the edges reflected near-UV rays (λ_{max} = 350 nm), whereas the flower center absorbed UV rays (Figure 4). Furthermore, a UV photograph clearly showed the existence of a conspicuous UV nectar guide in the central part of the corolla (Figure 5).

DISCUSSION

Headspace volatiles of *B. rapa* flowers, which consist exclusively of aromatic compounds, appear to have a simpler composition than those of the related

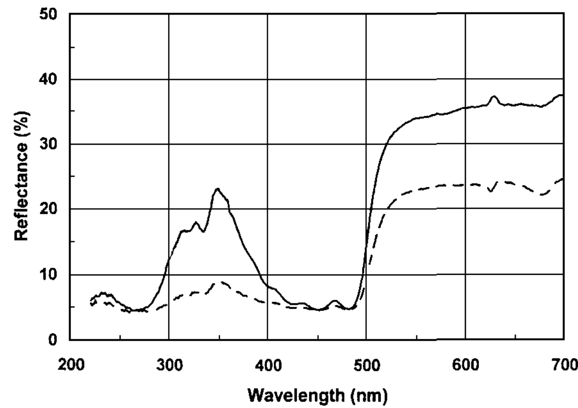

FIG. 4. Reflection spectra of a petal of rape flower, *B. rapa*. Solid line: outer edge of the petal; broken line: central part of the petal.

species, *B. napus*, which contain several terpenoids as additional constituents (Tollsten and Bergström, 1988). It is apparent that certain components of rape flower, e.g., phenylacetaldehyde and 2-phenylethanol, evoke definite PER from *P. rapae* adults, although the overall intensity of the response was not strong. There may be several causal factors underlying these insufficient responses in PER assays. For example, *P. rapae* was very susceptible to changes in atmospheric conditions caused by flowing air, and some individuals responded very poorly to odor alone, which may reflect their high reliance on visual cues in flower recognition. At any rate, these factors might have caused considerable reduction in PER responsiveness. The importance of some aromatic compounds in foraging behavior has been reported for honeybees, *Apis mellifera*, which

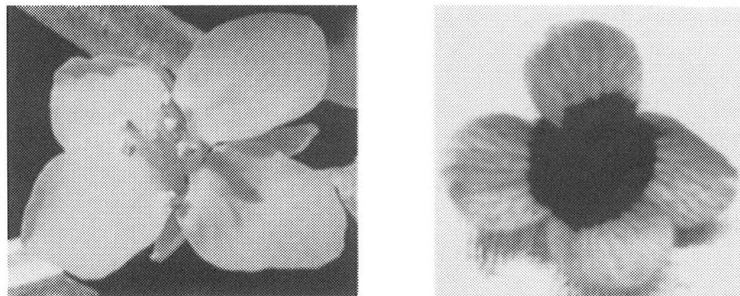

FIG. 5. Monochrome (left) and ultraviolet (right) photographs of rape flower, *B. rapa*.

showed conditioned proboscis extension in response to phenylacetaldehyde and 2-phenylethanol present in oilseed rape flower, *B. napus* (Blight et al., 1997).

In experiments with artificial flowers, five PER-active components, i.e., benzyl alcohol, 2-phenylethanol, benzaldehyde, phenylacetaldehyde, and phenylacetonitrile, significantly stimulated flower-visiting singly, whereas indole, which elicited no appreciable PER, was almost inactive in attraction. These data suggest close relationships between PER activity and the foraging-stimulatory activity of compounds and further indicate the usefulness of PER tests in elucidating potential compounds that may function as significant olfactory cues in foraging by butterflies. A synthetic blend of floral scents, although tested at a much smaller dose (1/200 of other compounds tested singly), was as effective as phenylacetaldehyde or 2-phenylethanol in attracting butterflies, and the data suggest that these specific floral components synergistically stimulate flower location by *P. rapae*. All these results are in good agreement with those obtained from our previous work that addressed the interaction between *P. rapae* and *L. japonicum* flowers (Honda et al., 1998). Phenylacetaldehyde, 2-phenylethanol, and benzaldehyde are compounds frequently encountered in the floral scents of many plant species (Knudsen et al., 1993). Wide distribution of these active substances in the plant kingdom may, in part, account for the broad spectrum of flowers visited by *P. rapae*.

EAG responses at a dose of 1 μg were generally weak, and differences in intensity were not remarkable among the compounds tested. EAG responses at 10 and 100 μg doses were positively correlated with PER performance (r = 0.822 and 0.793 for 10 and 100 μg, respectively) and stimulatory activities in flower-visiting. Although it is extremely difficult to determine the actual concentration at which foraging butterflies are exposed to floral volatiles in the natural environment, our data seem to imply that floral scents do not act as long-range attractants but rather as short-range cognitive cues that help butterflies detect and select flowers on which to forage.

The corolla of *B. rapa* flower is bright yellow, for which foraging adults of *P. rapae* have been reported to show strong preference (Miyakawa, 1976). In addition to this feature, the corolla of the flower was found to have a conspicuous nectar guide in its center, which, in contrast to the other areas, specifically absorbed near-UV rays in the range of 290–400 nm (λ_{max} = 350 nm). Inflorescences of some other crucifers such as *Brassica oleracea*, *Sinapis alba*, and *Rorippa indica* also are known to have similar UV nectar guides (Horovitz and Cohen, 1972; Utech and Kawano, 1975; Tanaka, 1982). It has been reported that nectar guides play a significant role in the foraging behavior of *P. rapae* (Kandori and Ohsaki, 1998).

The present study clearly demonstrates that adults have an innate preference of their own for particular odors and that the floral scent of *B. rapa* in itself strongly stimulates flower-visiting by *P. rapae*. As a result, it may be concluded

that olfactory and visual attractiveness of *B. rapa* flowers are most responsible for preferential visiting by *P. rapae*. Moreover, *B. rapa* is also one of the major host plants utilized by *P. rapae* in spring. Therefore, the butterfly's contribution to the reproductive success of the plant through pollination would in turn eventually ensure sufficient food for the butterfly species itself the following spring.

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JASMONIC ACID AND HERBIVORY DIFFERENTIALLY INDUCE CARNIVORE-ATTRACTING PLANT VOLATILES IN LIMA BEAN PLANTS

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Abstract—Lima bean plants respond to feeding damage of two-spotted spider mites (*Tetranychus urticae*) with the emission of a complex blend of volatiles that are products of several different biosynthetic pathways. These volatiles attract the carnivorous mite *Phytoseiulus persimilis*, a specialist predator of the spider mites that exterminates entire prey populations, and thus the volatiles contribute indirectly to plant defense. The volatile blend constitutes information to the carnivores, and blend composition is an important factor in this. Jasmonic acid (JA) is involved in the signal transduction of this induced defense. Application of JA through the petiole of Lima bean plants induces a volatile blend that is similar, but not identical, to that emitted by spider mite-infested plants. The induced volatiles originate from the lipoxygenase pathway, the shikimic acid pathway, and the isoprenoid pathway. Among the induced bean plant volatiles are nitriles and oximes. Of a total of 61 components, 10 are emitted at significantly different rates. Among these are the terpene (*E*)-4,8-dimethyl-1,3,7-nonatriene and the phenolic methyl salicylate, two compounds that are known to attract *P. persimilis*. A crucial test for comparing the effect of spider mite damage and JA application on volatile induction is the response of *P. persimilis*. The carnivore is attracted by volatiles from JA-treated plants. Moreover, even treatment of Lima bean plants with methyl jasmonate vapor made the plants attractive to the carnivorous mites. However, the predators prefer the volatiles from spider-mite-infested Lima bean plants over those from JA-treated plants. Thus, chemical as well as behavioral analyses demonstrate that spider mite damage and JA treatment have similar, although not identical, effects on volatile induction in Lima bean plants.

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Key Words—Jasmonic acid, carnivore attraction, Tetranychus, Phytoseiulus, terpenes, oximes, nitriles, methyl salicylate, induced defense.

INTRODUCTION

It has long been known that plant defense can be induced by wounding or herbivory (see Karban and Baldwin, 1997). Most knowledge on the induction of plant defense and the signal transduction involved is available for direct defense through plant characteristics that negatively affect herbivore performance. Examples are the induction of protease inhibitors or nicotine in response to wounding (Farmer and Ryan, 1992; Karban and Baldwin, 1997). These plant products negatively affect the herbivore's physiology and may result in herbivore death or retarded development. In addition, plants can also respond to herbivory with the production of volatiles that attract carnivorous natural enemies of the herbivore. This so-called indirect defense is a common type of plant defense against herbivorous arthropods (Dicke, 1999). Indirect defense affects animal behavior: the induced plant volatiles constitute information that is used in foraging decisions by carnivorous arthropods when searching for herbivorous prey (Turlings et al., 1990, 1993a; Dicke et al., 1990; Dicke, 1999). Behavioral responses of carnivorous arthropods to herbivore-induced plant volatiles are flexible and the composition of the blend of volatiles can have a great impact (Takabayashi et al., 1991; Turlings et al., 1993a; Vet et al., 1998). Changes in ratios of blend components can affect whether a carnivorous mite is attracted or not (Sabelis and van de Baan, 1983; Sabelis and Dicke, 1985; Takabayashi et al., 1991). Therefore, in studying indirect defense, the demonstration of the presence of a compound is a first step to show that a certain treatment induces defense, but it is not sufficient. The ultimate evidence needed is a demonstration that the carnivore responds to the induced volatile blend.

One of the systems for which induced indirect defense has been well studied is that of Lima bean (*Phaseolus lunatus*) plants, the herbivorous spider mite (*Tetranychus urticae*), and the predatory mite (*Phytoseiulus persimilis*). In response to spider mite damage, de novo production is induced of several terpenoids, such as (*E*)-β-ocimene, linalool, and the two homoterpenes (*E*)-4,8-dimethyl-1,3,7-nonatriene and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene as well as the phenolic methyl salicylate (Dicke et al., 1990; Boland et al., 1995; Bouwmeester et al., 1999). These compounds are not induced by mechanical damage alone. Volatiles from spider-mite-infested Lima bean plants are highly attractive to the predatory mite *P. persimilis*, in contrast to volatiles from mechanically damaged Lima bean leaves. The spider-mite-induced volatiles (*E*)-β-ocimene, linalool, (*E*)-4,8-dimethyl-1,3,7-nonatriene, and methyl salicylate play a role in this attraction (Dicke et al., 1990). However, their mere presence

does not always lead to attraction of the predator *P. persimilis*, but is dependent on total composition of the volatile blend. Gerbera plants infested with *T. urticae* emit all four above-mentioned predator attracting compounds in addition to a large number of other compounds, but do not attract *P. persimilis*, unless the predators have experienced the blend from spider-mite infested gerbera plants (Krips et al., 1999).

Studies on signal transduction in induced indirect defense have taken a lead from the vast knowledge on induced direct defense (e.g., Farmer and Ryan, 1992; Bowles et al., 1994; Wasternack and Parthier, 1997; Karban and Baldwin, 1997). Three major signal transduction pathways can be distinguished: (1) the octadecanoid pathway with jasmonic acid (JA) as the central component (Farmer and Ryan, 1992; Wasternack and Parthier, 1997), (2) the phenolic pathway with salicylic acid as the central component (Metraux et al., 1990; Malamy et al., 1996; Ryals et al., 1995; Karban and Baldwin, 1997) and (3) the ethylene pathway (Laat and Van Loon, 1981; O'Donnell et al., 1996; Karban and Baldwin, 1997). Recent studies have indicated that the octadecanoid pathway is involved in induced indirect defense. Exposure of Lima bean plants to JA results in the production of several volatiles that are also emitted in response to spider mite feeding (Hopke et al., 1994; Boland et al., 1995). However, a comparison of the composition of the blends emitted by spider mite damaged and JA-treated plants has not yet been made. To study the extent to which the octadecanoid signaling pathway is involved in induced indirect defense of Lima bean plants, two questions need to be answered: (1) Is the composition of the volatile blend emitted by Lima bean plants in response to JA the same as the blend emitted in response to spider mite-infestation? This will reveal whether the volatile blends are similar in a chemical sense. (2) Are carnivorous enemies of spider mites attracted to plants treated with JA? If so, do predatory mites discriminate between the blends emitted by spider-mite infested and JA-treated plants? Answering this question will show how similar the volatile blends are for the predatory mites. In this paper we address these two questions.

METHODS AND MATERIALS

Plants and Mites

Lima bean plants (*Phaseolus lunatus*), cv. Sieva (Kellog Seed Co., Crows Landing, California, USA) were reared in a glasshouse at 20–30°C, 50–70% relative humidity and a photophase of at least 16 hr. If the light intensity dropped below 500 μmol photons/m^2/sec, supplementary artificial illumination was by high-pressure mercury lamps. Unless stated otherwise, the plants were reared in steam-sterilized soil.

Spider mites (*Tetranychus urticae*) were reared on Lima bean plants in another glasshouse compartment under the same conditions as used for rearing

the plants. Predatory mites (*Phytoseiulus persimilis*) were reared on Lima bean plants or detached Lima bean leaves infested with spider mites. This was done in similar environmental conditions to those used for the rearing of plants.

Plant Treatments

Jasmonic Acid Exposure. Young uninfested Lima bean plants with the primary leaves just unfolded were individually exposed to a JA solution. The stems were cut just above soil level with a razor blade, and the stem of each plant was immediately placed in a vial with 6 ml of a JA solution (Sigma-Aldrich). The vial opening was sealed with parafilm. Two concentrations were used: 0.1 and 1 mM, based on Hopke et al. (1994). The plants were placed in a plastic cage ($66 \times 66 \times 100$ cm) in a climate room at $23 \pm 1°C$, 60–70% relative humidity, 700 μmol photons/m^2/sec. Control uninfested plants were similarly treated, but they were placed in water in another plastic cage in the same climate room. Each cage was connected to house vacuum to remove volatiles emitted from the plants. After two days of incubation, five plants of treatment and control were used for bioassays with predatory mites or for collection of volatiles. The 6 ml of JA solution had been almost completely taken up by the plants.

Spider Mite Infestation. Young plants with the primary leaves just unfolded were cut just above soil level, and each plant was individually placed with its stem in a vial with water. Each plant was infested with 100 adult female spider mites (*T. urticae*). The spider mites do not immediately initiate feeding but move about before starting to feed. Therefore, for a comparison of spider-mite infestation and JA treatment, the duration of spider-mite infestation needed may be longer than the incubation with JA. Thus we compared the effect of two and three days of spider mite infestation to the effect of two days of JA incubation. The spider-mite-infested plants were incubated under the same environmental conditions as the JA-treated plants. Five spider-mite-infested plants were used for the collection of volatiles. In the Y-tube olfactometer, five spider-mite-infested plants vs. five JA-treated plants were offered as odor source to the predatory mites.

Methyl Jasmonate (MeJA) Exposure. About 35–40 young Lima bean plants with unfolding primary leaves, reared in vermiculite, were placed in a 10-liter glass container in a climate cabinet at $29 \pm 1°C$, light intensity 350 μmol photons/m^2/sec. Five cotton wicks with a solution of MeJA (Bedoukian Research Inc., Danbury, Connecticut, USA) in ethanol (in total 1 μl MeJA/100 μl ethanol per five wicks) were placed in the container. After a 6-hr incubation, the plants were removed from the container and placed in a climate cabinet under the same environmental conditions as during incubation. An equal number of control plants of the same age was similarly incubated but without MeJA solution.

After 1, 2, 4, 7, and 14 days, seven MeJA-treated and seven control plants were collected. Their stems were cut just above vermiculite level with a razor

blade, and the plants were immediately placed individually in a vial with water. These cut plants were used as odor sources in the Y-tube olfactometer to investigate the response of the predatory mites.

Y-Tube Olfactometer

In a closed-system glass Y-tube olfactometer, individual predatory mites were offered a choice between: (1) control plants and plants treated with MeJA or JA or (2) plants treated with JA and plants infested with spider mites. An iron Y-shaped wire was present in the center of the Y-tube. For more details of the olfactometer, see Takabayashi and Dicke (1992). Adult female predatory mites that had been starved for 1–3 hr were individually positioned on the iron wire. When a predator reached the end of one of the arms of the olfactometer, this was recorded as a choice. The mite was subsequently removed, and a new mite was introduced. The maximum time that each individual was allowed to make a choice was 5 min. After every five mites the two odor sources were interchanged to adjust for potential asymmetries in the experimental arena. A total of 20 predators was used per replicate experiment.

The behavioral responses were statistically analysed with a χ^2 test to determine whether their distribution differed from 50:50 ($\alpha = 0.05$). Mites that had not made a choice within 5 min were excluded from the statistical analysis.

Predator Time Allocation on JA-Treated vs. Control Plant

In the olfactometer experiment the predators were exposed to distant odors from treated plants. To investigate the effect of jasmonic acid treatment on predator behavior when the predators were actually walking on the foliage, we conducted the following experiment. In this experiment the predators are directly exposed to the odor-emitting tissue and they may perceive the chemicals both as volatile and nonvolatile by tapping with their chemosensors on the leaves (Dicke et al., 1991).

Individual *P. persimilis* females were allowed to move freely between two adjacent Lima bean plants, one uninfested plant treated with JA and one uninfested control plant. The two plants were connected by a T-shaped iron wire. The horizontal arm of the T-shaped wire was 10 cm long and touched a leaf from each of the two plants. The vertical arm of the T-shaped wire was 20 cm long. Adult female predatory mites (starved for 1–3 hr) were individually released at the base of the T-shaped iron wire. After the predator walked upwards and crossed a mark 8 cm from the release point, the time recording started. Predator location was continuously recorded for 30 min or until the predator left the setup. Total times on the JA-treated plant and on the control plant were recorded. The times spent on each of the two plants were statistically analyzed with a Wilcoxon-matched-pair-signed-rank test ($\alpha = 0.05$).

For each predatory mite (51 or 55 mites per experiment), a new set of plants

was used and the positions of the JA-treated and control plant were switched after every replication. The experiments were carried out at 22 ± 3°C.

Collection and Analysis of Headspace Volatiles

Headspace samples were collected in the period June–July 1997. Odor sources were contained in a 5-liter glass flask with a 10-cm-ID opening, that was closed by a glass lid with an air inlet and an air outlet. Plants were carefully removed from the vials in which they had been exposed to JA solution or water. They were then transferred to glass vials filled with tap water that were placed inside the 5-liter collection flasks. For each headspace collection, we used five plants. Plants had been exposed to 1 mM JA for two days, to tap water for two days, or they had been infested with 100 spider mites each for three days. For each of the three treatments, five independent headspace collections were made.

Pressurized air was filtered over silica gel, molecular sieves 4A and 13X (Linde), and activated charcoal before entering the flask. The air inlet, air outlet, filters and sampling jar were connected with 0.8-cm-diam Teflon tubing. The system was purged for 1 hr at an airflow rate of 500 ml/min to remove volatile contaminants. Subsequently, volatiles were collected on Tenax-TA for 30 min at a flow rate of 500 ml/min.

The collected volatiles were released from the Tenax by heating the trap in a Thermodesorption Cold Trap Unit (Chrompack) at 250°C for 10 min and flushing with helium flowing at 10 ml/min. The released compounds were cryofocused in a coldtrap (0.52 mm ID deactivated fused silica) at a temperature of −85°C. By ballistic heating of the cold trap to 220°C the volatiles were transferred to the analytical column (Supelcowax 10, 60 m × 0.25 mm ID, 0.25-μm film thickness), which was connected to a Finnigan MAT 95 mass spectrometer. The column was programmed from 40°C (3 min hold) to 270°C (4 min hold) at 4°C/min and the initial helium velocity was 30 cm/sec. The mass spectrometer was operated in the 70 eV EI ionization mode and scanning was done from mass 24 to 400 at 0.7 sec/decade. Compounds were identified by comparison of the mass spectra with those in the Wiley library and in the Wageningen Mass Spectral Database of Natural Products and by checking the retention index. Emission rates were measured by quantifying peak areas and compared statistically over treatments with the Mann-Whitney U test ($\alpha = 0.05$). To obtain an absolute impression of emission rates, we made a calibration for a set of very different compounds. One peak area unit represents 0.17 ± 0.05 ng.

RESULTS

Composition of Induced Volatile Blends

Uninfested Lima bean plants emitted limited amounts of volatiles, while JA-treated and spider-mite-infested plants emitted, on average, 30–50 times larger

amounts. The total amount of volatiles emitted by spider-mite-infested and JA-treated plants was similar ($P > 0.05$, Mann-Whitney U test) (Table 1). The headspace composition of spider-mite-infested Lima bean plants is similar, but certainly not identical to that of Lima bean plants incubated in 1 mM JA (Table 1). Most components are emitted at rates that do not significantly differ. Ten compounds, however, have emission rates that do differ significantly between JA-treated and spider-mite-infested plants: (*Z*)-4,8-dimethyl-1,3,7-nonatriene, (*E*)-4,8-dimethyl-1,3,7-nonatriene, (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, another 4,8,12-trimethyl-1,3,7,11-tridecatetraene isomer, methyl salicylate and two unidentified compounds (with mass spectra 41,69B,79,95,109,149, 164, and 43B,79,93,94,148,151,166), are emitted in larger quantities by spider-mite-infested plants than by JA-treated plants, while 2-methylpropanal-*O*-methyloxime, 2-methylbutanal-*O*-methyloxime, and (*Z*)-3-hexen-1-ol 2-methylbutanoate are emitted in larger amounts by JA-treated plants (Table 1). Among these compounds, (*E*)-4,8-dimethyl-1,3,7-nonatriene and methyl salicylate are of special interest because they are known to attract *P. persimilis* (Dicke et al., 1990). Thus, JA treatment and spider mite infestation of Lima bean plants led to blends of volatiles that appear qualitatively similar, but that differ substantially in the contribution of a number of components (Table 1).

Attraction of Predatory Mites

Treatment of Lima bean plants with a 1 mM JA solution resulted in significant attraction of predatory mites. In 14 of 15 replicate olfactometer tests, the majority of predators preferred the odor of the treated plants. Overall 163 predators chose the odor of JA-treated plants vs. 94 choosing the odor of uninfested control plants (Figure 1). Incubation in 0.1 mM JA solution did not lead to a significant preference of the predatory mites in the olfactometer (Figure 1). The volatile blend from spider-mite-infested plants was more attractive than that of JA-treated plants. The predators preferred the volatile blend from spider-mite-infested plants over the blend from 1.0 mM JA-treated plants (Figure 1).

In a two-choice experiment where predators could walk on a JA-treated and a control plant for 30 min, predatory mites spent more time on the undamaged JA-treated plant than on the undamaged control plant. This difference was statistically significant for the 0.1 mM treatment vs. the uninfested control, but marginally not significant for the 1.0 mM treatment vs. the uninfested control (Figure 2). The distributions of initial choices of the predators were similar to those observed in the olfactometer. When a 1.0 mM JA-treated plant was offered vs. a control, 37 predators first chose the JA-treated plant and 18 the control plant (χ^2 test, $P = 0.01$). In contrast, when a 0.1 mM JA-treated plant was offered vs. a control plant, 27 predators first chose the JA-treated plant and 24 the control plant (χ^2 test, $P = 0.67$).

TABLE 1. COMPOSITION OF HEADSPACE OF 5 LIMA BEAN PLANTS TREATED WITH 1 mM JASMONIC ACID SOLUTION, INFESTED WITH TWO-SPOTTED SPIDER MITES (*T. urticae*) OR CONTROL PLANTS[a]

Compound	Jasmonic acid treatment	Spider mite infested	Control	P(JA va spider mite infested)[b]
Nitrogenous compounds				
1 2 methyl-propanal-*O*-methyloxime	65.8 (31–114)	15.2 (0–30)	0.4 (0–2)	0.012
2 2 methyl butanal-*O*-methyloxime	311.8 (216–536)	59.2 (0–140)	0 0	0.012
3 3 methyl butanal-*O*-methyloxime	163.8 (6-376)	28.8 (0–52)	6.2 (0–10)	NS
4 2 methyl propane nitrile	23.8 (10–43)	10.6 (0–28)	1 (0–5)	NS
5 2-methylbutanenitrile	11 (0–38)	1.4 (0–5)	0 0	NS
6 3-methylbutanenitrile	122.6 (8–334)	12.8 (0–28)	0 0	NS
7 2-pentenenitrile oi	7.2 (0–18)	0 0	0 0	NS
8 rose furan	19.8 (0–51)	5.2 (0–26)	0 0	NS
Aldehydes				NS
9 2 methyl-2-propenal	29.6 (0–82)	18.2 (0–51)	0 0	NS
10 hexanal	7.4 (0–20)	13.4 (0–62)	0 0	NS
11 (*E*)-2-hexenal	25.4 (0–70)	6.6 (0–33)	0 0	NS
12 octanal	36.8 (0–102)	41 (0–131)	0 0	NS
13 nonanal	117 (44–285)	123.8 (23–274)	31 (0–68)	NS
14 decanal	323.6 (0-697)	240 (35–518)	64.6 (35–133)	NS
Ketones				NS
15 2-butanone	247.4 (93–297)	313.2 (216–498)	1.2 (0–6)	NS
16 3-butene-2-one	14.8 (0–50)	11.4 (0–19)	0 0	NS
17 3-pentanone	29.8 (16–49)	39.8 (33–44)	11 (0–23)	NS
18 3 methyl 3-buten-2-one	11.8 (0–43)	4.2 (0–21)	0 0	NS
19 1-penten-3-one	4.4 (0–12)	0 0	0 0	NS
20 3-heptanone	0.6 (0–3)	25.4 (0–78)	4.2 (0–21)	NS
21 3-octanone	8.3 (0–11)	0.8 (0–4)	4.6 (0–23)	NS
Alcohols				NS
22 1-butanol	3.4 (0–11)	0 0	13.4 (0–26)	NS
23 2-butanol	6.6 (0–19)	1.6 (0–8)	0 0	NS
24 2 methyl 3-buten-2-ol	10.2 (0–39)	2.8 (0–14)	0 0	NS
25 1-penten-3-ol	2.6 (0–8)	1.8 (0–9)	0 0	NS
26 1-pentanol	0 0	9.2 (0–46)	1.2 (0–6)	NS
27 (*Z*)-3-hexen-1-ol	95.2 (37–134)	169.4 (61–463)	12.2 (0–47)	NS
28 1-octen-3-ol	21.8 (0–65)	27.8 (0–96)	10.2 (0–34)	NS
29 1-nonanol	10.4 (0–36)	14.8 (0–38)	0 0	NS

Esters				NS
30 3-methyl-1-butanol acetate	6 (0–18)	0 0	0 0	NS
31 pentyl acetate	3.2 (0–9)	1.4 (0–7)	0 0	NS
32 hexyl acetate	40 (11–96)	16.8 (0–47)	0 0	NS
33 (*E*)-3-hexen-1-ol acetate	1.4 (0–7)	0 0	0 0	NS
34 (*Z*)-3-hexen-1-ol acetate	1133.8 (318-2060)	1435.4 (997–1900)	17 (0–58)	NS
35 (*E*)-2-hexen-1-ol acetate	11.6 (0–58)	0 0	0 0	NS
36 (*Z*)-3-hexen-1-ol butanoate	11.4 (0–33)	3 (0–15)	0 0	NS
37 (*Z*)-3-hexen-1-ol 2-methylbutanoate	102.4 (47–216)	39.6 (18–74)	0 0	0.047
Terpenes				
38 myrcene	11.8 (0–46)	5.2 (0–15)	0 0	NS
30 (*Z*)-β-ocimene	147.8 (30–361)	93.4 (19–158)	0 0	NS
40 (*E*)-β-ocimene	1881.8 (523–3880)	1460.4 (603–2480)	2 (0–7)	NS
41 (*Z*)-4,8-dimethyl-1,3-7-nonatriene	1.6 (0–8)	42.4 (15–62)	0 0	0.012
42 (*E*)-4,8-dimethyl-1,3-7-nonatriene	457.2 (314–684)	2418 (1360–3710)	35 (15–58)	0.012
43 allo-ocimene	2.6 (0–13)	0 0	0 0	NS
44 α-copaene	12.2 (0–28)	37.6 (6–68)	0 0	NS
45 linalool	259.2 (67–846)	181.4 (83–354)	5.4 (0–22)	NS
46 α-bergamotene	33.6 (0–133)	0 0	0 0	NS
47 β-caryophyllene	244 (80–342)	268 (0–502)	1.8 (0–9)	NS
48 4,8,12-trimethyl-1,3,7,11-tridecatetraene isomer	0 0	60.4 (0–96)	0 0	0.025
49 (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene	18.8 (11–26)	1796 (1000–2570)	2.1 (0–6)	0.012
Others				
50 methyl salicylate	20 (5–37)	2008 (1450–2780)	6.4 (0–15)	0.012
51 jasmone	2.8 (0–14)	0 0	0 0	NS
52 benzyl cyanide	569.2 (14–1700)	75.8 (0–180)	0.3 (0–2)	NS
53 indole	229.4 (50–519)	140.2 (77–251)	7.8 (0–15)	NS
Unidentified				NS
54 unidentified 55,83,84B	2 (0–10)	15.6 (0–37)	0 0	NS
55 unidentified 91B, 107, 135	17.8 (0–39)	17.4 (0–65)	0 0	NS
56 unidentified 91,93,95B,150	48.2 (0–151)	34.6 (0–78)	0 0	NS
57 unidentified 41,55,69,70B,83,134	69 (0–202)	35.2 (0–141)	0 0	NS
58 unidentified 41,69B,79,95,109,149,164	16 (0–80)	205.6 (65–420)	0 0	0.022
59 unidentified 41,69B,107,147,218	0 0	25 (0–73)	0 0	NS
60 unidentified 43B,79,93,94,148,151,166	27.2 (0–86)	172.8 (51–310)	0 0	0.021
61 unidentified 67,71,82B	54.6 (0–200)	6 (0–30)	0 0	NS
Total	7167.8 (2883—13106)	11793.6 (7740–16636)	239 (161–287)	

[a] Plants of all treatments had been cut just above soil level three days before collection of volatiles and placed in tap water or JA solution at 23 ± 1°C, 60 ± 10% relative humidity, 700 μmol photons/m^2/sec. $N = 5$ for all three treatments, headspace collection 30 min at 500 ml/min. For each treatment the average amount is indicated and the range in terms of peak area units is in parentheses. 1 peak area unit is 0.17 ± 0.05 ng.
[b] For each compound the peak areas are statistically compared for the JA treatment vs. the spider mite infestation treatment with a Mann-Whitney U test ($\alpha = 0.05$).

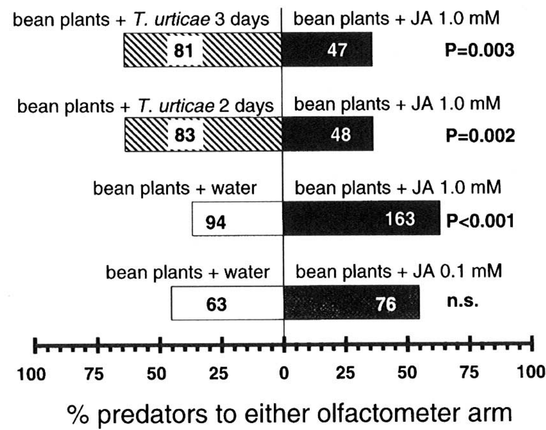

FIG. 1. Response of adult female carnivorous mites (*P. persimilis*) in a Y-tube olfactometer when offered five Lima bean plants exposed to 0.1 mM or 1.0 mM jasmonic acid for two days vs. five Lima bean plants exposed to water for two days or when offered five Lima bean plants exposed to 1.0 mM jasmonic acid for two days vs. 5 Lima bean plants infested by spider mites (*T. urticae*) for either two or three days. Predators that did not choose either arm are not included in the graph. Numbers in bars represent numbers of carnivores that made the corresponding choice. *P* values refer to χ^2 test; NS: $P > 0.05$.

Treatment of intact Lima bean plants with MeJA vapor resulted in attraction of the predatory mite *P. persimilis*. The predators significantly preferred the odor from MeJA-treated plants over odor from control plants at two and four days after treatment. At 1, 7, and 14 days after treatment more than 50% of the predators chose the odor of treated plants, but there was no significant difference

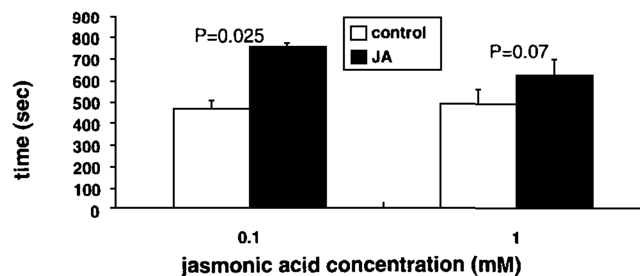

FIG. 2. Time allocation (mean ± SE) of adult female carnivorous mites in a two-choice experiment on leaves of a Lima bean plant exposed to jasmonic acid vs a control plant. $N = 51$ for 0.1 mM JA vs. control and $N = 55$ for 1 mM JA vs. control.

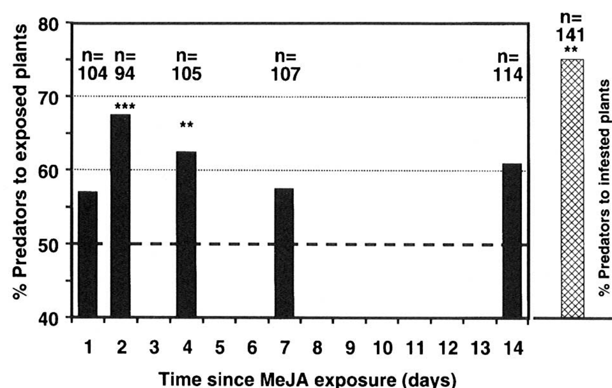

FIG. 3. Response of adult female carnivorous mites (*P. persimilis*) in a Y-tube olfactometer when offered seven Lima bean plants exposed to methyl jasmonate (MeJA) vs. seven control plants. As a comparison, the response of predators from the same population to odors from nine Lima bean leaves infested with spider mites vs. uninfested Lima bean leaves is presented. Predators that did not choose either arm are not included in the graph. n indicates the number of mites that made a choice. ***$P < 0.001$; **$0.001 \leq P < 0.01$ (χ^2 test).

from a 50 : 50 distribution (Figure 3). The strongest degree of attraction occurred 2 days after treatment (Figure 3).

DISCUSSION

This study shows that JA treatment and spider mite infestation induce similar, although not identical, responses in Lima bean plants. Chemical analyses show significant differences in headspace composition, and carnivorous enemies of the spider mites discriminate between the blends from JA-treated and spider-mite-infested plants.

JA induces the emission by Lima bean plants of volatiles that originate from very different biosynthetic pathways, such as the lipoxygenase pathway [e.g., hexanal, (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol], the shikimic acid pathway (methyl salicylate), and the isoprenoid pathway [e.g. (*E*)-*β*-ocimene, (*E*)-4,8-dimethyl-1,3,7-nonatriene, linalool]. This is in accordance with reports that JA induces enzymes involved in the lipoxygenase and shikimic acid pathway (Avdiushko et al., 1995; Bell and Mullet, 1991; Lois et al., 1989). Our study is the first to demonstrate that nitrogenous products such as oximes and nitriles are induced by JA. These compounds are likely produced from amino acids (Kaiser, 1993). The simultaneous induction by JA of several biosynthetic pathways leads to a complex blend of novel volatiles that are not emitted by control Lima bean plants.

The volatile blend emitted by Lima bean plants after incubation in 1 mM JA solution is similar to that from spider-mite infested Lima bean plants: 51 of 61 compounds are emitted in similar amounts. The composition differs significantly, however, with regard to the relative amounts of 10 compounds, among which are two known to mediate attraction of the predatory mite *P. persimilis*. The predator attractant (*E*)-4,8-dimethyl-1,3,7-nonatriene (Dicke et al., 1990) is emitted at a ca. 5 times higher rate from spider-mite-infested plants than from JA-treated plants (Table 1). Moreover, the predator attractant methyl salicylate (Dicke et al., 1990) is emitted at a 100 times higher rate from spider-mite-infested plants (Table 1). The emission of methyl salicylate has been suggested to be a mechanism to dispose of induced salicylic acid in virus-infested tobacco plants (Shulaev et al., 1997). Salicylic acid and methyl salicylate are inhibitors of JA (Doares et al., 1995; Pena-Cortes et al., 1993; Baldwin et al., 1997). Because methyl salicylate is emitted at high rates by spider-mite-infested plants but not by JA-treated plants, its induction is likely to be JA-independent.

The comparison of headspace composition of JA-treated and spider-mite-infested plants leads to the conclusion that, although the response to JA resembles the response to spider mite damage, JA cannot be the only factor involved in the induction of indirect defense in Lima bean. Other factors may be, for example, endogenous components of other signal transduction pathways or the simultaneous presence of upstream precursors of JA together with JA in the plant (Farmer et al., 1998; Laudert and Weiler, 1998).

Behavioral analyses demonstrate that treatment of Lima bean plants with JA results in the attraction of predatory mites and in an increased search time of the carnivores on treated plants compared to untreated control plants. When the predators were offered a choice from a distance to the volatiles from JA-treated plants they were attracted to volatiles from plants exposed to 1 mM JA, but not to those emitted by plants that had been incubated with 0.1 mM JA. This was true both in the olfactometer and with regard to the first choice of the predators in the time-allocation experiment. Moreover, in the latter experiment an effect of treatment with 0.1 mM solution was seen on time allocation of predators. This shows that changes in plants exposed to 0.1 mM JA can affect the predators. Most likely, concentrations of volatiles emitted by plants treated with 0.1 mM JA were too low (Hopke et al., 1994) to be effective at long range. In contrast, at short range the chemoreceptors on the predators' front legs (Jagers op Akkerhuis et al., 1985) are close to the source of the volatiles. There, concentrations may be sufficiently high to yield a response (Dicke et al., 1991) or, alternatively, the compounds may be perceived by contact chemoreceptors on the pedipalps that tap the plant surface (Jagers op Akkerhuis et al., 1985; Dicke et al., 1991).

Despite the response to volatiles emitted by JA-treated plants, the carnivores prefer the volatiles from spider-mite infested plants over those from JA-exposed plants. This demonstrates that the chemical differences in volatile blends

between plants infested by spider mites and plants treated with JA are biologically important and thus that the effect of spider mite infestation cannot be fully explained by JA application. This observation emphasizes the importance of studying carnivore behavior in addition to making a chemical analysis of the headspace composition.

Moreover, exposure of intact Lima bean plants to MeJA vapor also resulted in attraction of *P. persimilis*, although it had been reported that exposure to MeJA does not result in the induction of homoterpenes and thus not in the induction of indirect defense (Hopke et al., 1994). This is one more example of a response of an animal to a stimulus that does not evoke a response from an analytical detector (see also Schütz et al., 1997; Weissbecker et al., 1997), which underlines the importance of making biological analyses in conjunction with chemical analyses. The chemoreceptors in the animals are likely more sensitive than the detector of the analytical equipment. More sensitive assays to quantify the induction of volatile production in response to MeJA are needed in the future. Monitoring induced expression of regulating genes in the induced pathways (Bouwmeester et al., 1999) may be an important tool for this.

The composition of herbivore-induced plant volatile blends can vary with different herbivore species that damage the plant, and carnivores discriminate between these blends (Sabelis and van de Baan, 1983; Takabayashi et al., 1991; Turlings et al., 1993a; Powell et al., 1998; for review see Dicke, 1999). Elucidating what mechanisms are used by plants in the specific responses to different herbivores remains an exciting task for future studies. Oral secretions of caterpillars can induce plant responses that are similar to the response to caterpillar damage (Turlings et al., 1993b; Mattiacci et al., 1995). In each case, a single component of these oral secretions can effectively mimic the effect of the oral secretion or caterpillar feeding damage (Mattiacci et al., 1995; Alborn et al., 1997). This suggests that a single herbivore elicitor initiates the signal transduction pathway(s). If that is true for spider mite–Lima bean interactions as well, the specificity of the volatile blends emitted should be caused by a combination of endogenous elicitors, of which JA is one. Exogenous elicitors are known to induce endogenous elicitors such as JA in plants (Gundlach et al., 1992; Baldwin et al., 1997). Which other induced endogenous elicitors play a role remains to be investigated.

The effect of MeJA exposure of Lima bean plants on attraction of predatory mites demonstrates that a plant can respond to a volatile elicitor with the production of carnivore attractants. This is important with respect to the demonstration that exposure of Lima bean plants to the volatiles from upwind spider-mite-infested plants results in attraction of carnivorous mites to the exposed plants (Bruin et al., 1995). Those results can be explained by a passive mechanism, i.e., contamination of the exposed plants with predator attractants and subsequent second-hand release, or an active mechanism, i.e., communication where an elicitor of infested

plants induces a response in the exposed plant (Bruin et al., 1995). Our data show that a volatile elicitor can induce a carnivore-attracting volatile blend in undamaged Lima bean plants that resembles the induction of direct defense in tomato (Farmer and Ryan, 1990). The MeJA-induced attraction cannot be explained by a response of the predatory mites to MeJA itself. If MeJA were attractive, the response to MeJA-treated plants would be expected to decline with time since MeJA exposure. In contrast, we found that one day after MeJA exposure the response was not statistically significant, while the response is highly significant two days after exposure. Thus, our results suggest that plant-to-plant communication may result in the emission of carnivore attractants in plants next to spider-mite-infested plants. Our data show that direct and indirect defenses of plants against herbivores share a common signal transduction pathway through JA. Yet, the response of plants to different herbivore species or to wounding may be specific for the damaging organism. Elucidating how plants respond differentially to different types of damage is a challenge for future studies.

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LEAF VOLATILES FROM NONHOST DECIDUOUS TREES: VARIATION BY TREE SPECIES, SEASON AND TEMPERATURE, AND ELECTROPHYSIOLOGICAL ACTIVITY IN *Ips typographus*

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Abstract—The leaf volatiles emitted from four nonhost tree species of *Ips typographus*, i.e. *Betula pendula*, *B. pubescens*, *Populus tremula*, and *Sambucus nigra*, were collected outdoors by headspace sampling in situ and analyzed by GC-MS. Three major classes of compounds, aliphatics [mainly green-leaf volatiles (GLVs)], monoterpenes, and sesquiterpenes, existed in all the deciduous tree species investigated. In June, when the bark beetles are searching in flight for host trees, GLVs mainly consisting of (Z)-3-hexenyl acetate and (Z)-3-hexen-1-ol were the dominant constituents in *B. pendula* and *S. nigra*. In *B. pubescens* and *P. tremula*, sesquiterpenes (and their derivatives) and monoterpenes made up the major part of whole volatile blends, respectively. Surprisingly, sesquiterpene alcohols and other oxides released from *B. pubescens* in considerable amounts were not found in the closely related species, *B. pendula*. By August, both the total volatiles and individual compounds significantly decreased, mainly due to the maturation of leaves, since the light intensity and temperatures during sampling were the same as in June. There were almost no volatiles detected from *P. tremula* and *S. nigra* leaves in August. The total emissions from these deciduous species were significantly different among the species, with *B. pubescens* releasing 5–10 times more than other species. Under the conditions of

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constant light intensity and humidity, emissions of both total volatiles and most individual components of severed *B. pendula* and *S. nigra* branches (with fresh leaves) increased according to a saturation curve from 16°C to 40°C. *Ips typographus* antennae responded strongly to green leaf alcohols: (*Z*)-3-hexen-1-ol, 1-hexanol, and (*E*)-2-hexen-1-ol, but not to aldehydes or acetates in GC-EAD analyses of *B. pendula* and *B. pubescens* leaf volatiles. No antennal responses to monoterpenes, sesquiterpenes, or sesquiterpene oxides were found. These three antennally active GLVs emitted from nonhost tree leaves might be indicators of a wrong habitat in the host selection of conifer bark beetles.

Key Words—Birch, *Betula pendula* (=*verrucosa*), *B. pubescens*, Betulaceae, aspen, *Populus tremula*, Salicaceae, elder, *Sambucus nigra*, Caprifoliaceae, green-leaf volatiles, monoterpene, sesquiterpene, (*Z*)-3-hexen-1-ol, 1-hexanol, (*E*)-2-hexen-1-ol, seasonal variation, temperature effect, host selection, habitat, *Ips typographus*, Coleoptera, Scolytidae.

INTRODUCTION

The eight-spined spruce bark beetle, *Ips typographus*, is one of the most destructive forest insects in Europe and northern Asia (Postner, 1974). Mass attacks on standing and healthy spruce trees are believed to be initiated by an attractive signal, the aggregation pheromone produced by the pioneer males when boring into suitable host trees (Bakke, 1970; Birgersson et al., 1984; Schlyter and Löfqvist, 1986, Schlyter et al., 1987b). Either by primary attraction (Austarå et al., 1986; Lindelöw et al., 1992) or random landing (Schlyter and Birgersson, 1999), pioneer males find suitable hosts of Norway spruce, *Picea abies*. However, attraction to pheromone is reduced in the presence of decadent or fully colonized hosts, due to the release of volatiles such as verbenone that act in density regulation and host suitability signals (Bakke, 1981; Schlyter et al., 1987a, 1989). Guerrero et al. (1997) reported that benzyl alcohol, a nonhost compound, inhibited the positive responses of *Tomicus destruens* (Woll.) to pine volatiles. Host selection in phytophagous insects has long been hypothesized to involve both attraction to the host and avoidance of nonhosts (Visser, 1986; Byers, 1995).

Little is known about the mechanisms underlying rejection by bark beetles of nonhost tree species. Rejection could be based on a lack of host volatile characteristics or the presence of repellent or deterrent stimuli (Poland et al., 1998). Ambrosia beetles, *Trypodendron domesticum* and *Xyleborus (Anisandrus) dispar*, that attack broad-leaved trees respond negatively to α-pinene, one of the major monoterpene constituents of both Scots pine, *Pinus sylvestris*, and Norway spruce, *P. abies* (Nijholt and Schönherr, 1976; Schroeder and Lindelöw, 1989). In contrast, the coniferphagous bark beetles, *Dendroctonus frontalis*, *D. ponderosae*, *D. brevicomis*, *D. rufipennis*, *I. grandicollis*, *I. avulsus*, and the ambrosia beetles, *T. lineatum*, *Gnathotrichus sulcatus*, and *G. retusus*, avoid non-

host trees in part because they are repelled by green-leaf volatiles (GLVs), six-carbon primary alcohols, aldehydes, and derivative esters commonly found in green plants (Visser, 1986; Dickens et al., 1991, 1992; Wilson et al., 1996; Borden et al., 1997; Poland et al., 1998; Deglow and Borden, 1998a,b). Schlyter et al. (1995) found that *I. typographus*, *I. duplicatus*, and *Tomicus pinperda* were repelled by a blend of five GLVs and the terpene alcohol, linalool. In the presence of certain nonhost substances, *Scolytus multistriatus* rejected host-tree twigs suitable for maturation feeding (Gilbert and Norris, 1968). Attraction of both *T. piniperda* and *Hylurgops palliatus* to ethanol was reduced by odors from cut logs of nonhost birch (*Betula pendula*) and aspen (*Populus tremula*) trees (Schroeder, 1992). Single cell responses to the odor from birch bark have been demonstrated in *T. lineatum* and *I. typographus* (Tømmerås, 1989, Tømmerås and Mustaparta, 1989). Mountain pine beetles, *D. ponderosae*, were repelled in an additive and redundant manner by four antennally active volatiles from the bark of *Populus tremuloides:* 1-hexanol, benzylalcohol, benzaldehyde, and nonanal (Borden et al., 1998). Our recent study showed that attraction of *I. typographus* and *Pityogenes chalcographus* (L.) to their pheromone traps was significantly reduced by the presence of fresh birch bark and leaves (Byers et al., 1998).

European birches [*B. pendula* Roth. (=*B. verrucosa* Ehrh.) and *B. pubescens* Ehrh.], aspen (*P. tremula* L.) and elder (*Sambucus nigra* L.) are common deciduous tree species in Scandinavia and are often found in mixed stands with Norway spruce. Some of the volatiles released from these nonhost trees have been identified (Inki and Väisänen, 1980; Isidorov et al., 1985; König et al., 1995), but any effects on *I. typographus* have not been studied.

Our objectives were to: (1) identify and quantify the volatiles emitted from intact leaves of the above-mentioned nonhost tree species in both June and August; (2) study the effects of temperature on volatile emissions from severed branches with fresh leaves of *B. pendula* and *S. nigra* in laboratory; and (3) determine the electrophysiologically active compounds emitted from the nonhost trees by using coupled gas chromatographic-electroantennographic detection (GC-EAD).

METHODS AND MATERIALS

Collection of Volatiles. Volatiles released from intact leaves were collected from four nonhost trees species—*B. pendula*, *B. pubescens*, *P. tremula*, and *S. nigra*—in the field in southern Sweden by headspace sampling in June and August 1997 (Table 1). For each species, samples were taken from two neighboring trees of similar size. Three branches in June and two in August (35–40 cm long), at 1.5–1.7 m high were sampled separately from each experimental tree.

TABLE 1. IN SITU SAMPLING OF LEAF VOLATILES FROM FOUR ANGIOSPERM SPECIES IN THE FIELD, 1997, SOUTH SWEDEN[a]

Tree species	Date	Time (hr)	Tree height (m)	Locations	Leaves per sample (*N*)	Dry weight (g)	Temperature range (°C)	
							Inside bag	Outside bag
Betula pendula	June 2	11:15–13:45	3.5–4.0	Lomma	65–85	2.54–3.35	17.5–29.2	17.2–21.2
	August 5	12:00–14:00	3.5–4.0		61–96	4.25–5.40	24.4–30.1	21.9–23.1
B. pubescens	June 7	11:30–14:00	3.5–4.0	Dalby	80–100	2.64–4.40	26.4–38.0	24.0–38.4
	August 10	12:55–15:25	3.5–4.0		55–74	2.33–3.05	28.6–40.1	26.9–35.1
Populus tremula	June 5	13:00–15:30	4.5–5.0	Dalby	75–95	3.03–3.37	20.0–38.9	19.0–30.7
	August 6	12:55–15:25	4.5–5.0		100–140	5.25–5.40	22.1–30.7	20.4–23.0
Sambucus nigra	June 9	12:35–15:05	2.0–2.2	Alnarp	80–90	2.50–3.60	25.5–33.8	18.2–23.4
	August 4	12:45–15:15	2.0–2.2		42–43	4.27–6.40	24.2–31.1	21.9–24.0

[a]For each species, the same two trees were sampled in June and August with three and two branches per tree, respectively.

Intact branches with leaves were enclosed in a polyester cooking bag (Meny Toppits, Terinex Ltd, England, 35 × 43 cm) with a charcoal filter tube in the inlet. Coupled gas chromatographic–mass spectrometry (GC-MS) analysis showed no obvious release of volatiles from the blank bag. Air was drawn by vacuum for 2.5 hr over the branch of 150 ml/min and through a Teflon tube (3.0 mm ID × 35 mm) containing 30 mg of Porapak Q, mesh 50–80 (Supelco), with polypropylene wool and nylon stopper (1.68 mm ID) at both ends and connected by plastic tubing (4 mm ID, 80 cm long) to a mini-pump (5 × 6.5 × 3.5 cm; GroTech, Göteborg, Sweden) driven by a rechargeable battery (12 V) (Figure 1). The total weight of all equipment excluding the battery was only 450 g and with a sealed lead battery sufficient for several days of operation was <1.8 kg, which is significantly lighter than the apparatus Turgeon et al. (1998) used for capturing volatiles from conifer cones. Earlier versions of this setup were used effectively for capturing volatiles from individual insects (Birgersson and Bergström, 1989) and intact plants (Knudsen et al., 1993). The Porapak Q trap was attached to the branch by cotton twine and wrapped with aluminum foil to protect it from direct sunlight. Air temperatures inside and outside of sampling bags were recorded during the aerations with a digital Min-Max thermometer. After the aerations, the sample branches were cut, the leaves counted, and the fresh and dry (65°C, 72 hr) weight determined. Porapak Q traps were sealed with aluminum foil. An ambient air control sample was also collected for each of the sampling trees by using the same technique (without enclosure of the plastic bag) (see Figure 1).

As soon as possible after the field collection, the Porapak Q traps were

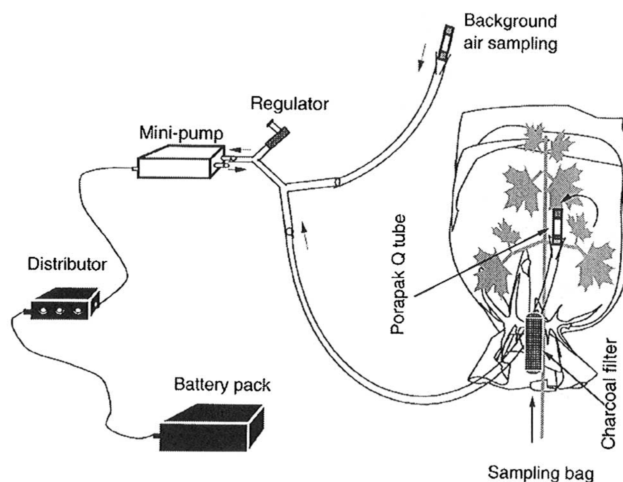

FIG. 1. Aeration setup for collection of volatiles from the intact leaves.

TABLE 2. SAMPLES FOR EXPERIMENT TESTING EFFECT OF TEMPERATURE DURING EX SITU SAMPLING IN 1997 ON VOLATILES FROM BRANCHES OF *B. pendula* AND *S. nigra* IN LABORATORY[a]

Species	Temperature (°C)		Leaves per sample	Dry weight	Date
	Inside bag	Outside bag	(*N*)	(g)	
B. pendula	16.7–17.3	15.7–16.4	100–130	4.16–5.71	May 30
	32.6–32.8	31.9–32.2	200–300	9.78–19.1	June 10
S. nigra	16.4–16.8	15.9–16.3	50–70	4.50–5.52	May 30
	24.9–25.2	24.4–24.8	50–70	3.60–5.31	May 28
	32.8–33.2	31.9–32.0	50–70	4.50–5.40	June 10
	39.2–39.9	39.1–39.6	105–150	6.67–18.02	June 11

[a]*N* = 5 branches per sample, light intensity held at 1000 lux.

rinsed with 300 μl diethyl ether (Fluka puriss p.a.) and collected into the insert tube (cone-shaped) of Hewlett Packard 2-ml vials for the GC autoinjector. Another 300 μl diethyl ether was added to the vial, outside of the insert tube, which prevented the eluate from evaporating from the insert. The sample vials were kept at −20°C. As an internal standard, 1 μg of octyl acetate was added to the June samples. No octyl acetate was added to the August samples because the diethyl ether stabilizer was found to be a satisfactory internal standard.

Aerations of newly severed branches of *S. nigra* and *B. pendula*, with the cut ends in water, were also carried out within 30 min after cutting at four and two temperature levels, respectively, with five branches per level (Table 2). Otherwise, procedures were similar to those used for in situ sampling.

Chemical Analysis. Chemical analyses were made by a HP 5890 series II gas chromatograph coupled with a HP 5972 mass selective detector (GC-MSD). The GC was equipped with a 25-m × 0.25-mm fused silica column, coated with CP-wax 58 (PEG). Samples were injected by using a HP 7673 auto injector (2 μl/injection). Helium was used as the carrier gas at a constant flow of 31 cm/sec at 50°C. The injector temperature was 200°C. Oven temperature for June and temperature effect samples was 50°C for the first 2 min, rising to 210°C at 10°C/min, and then held for 5 min, and for August samples was 30°C for the first 3 min, rising to 200°C at 10°C/min, and held for 2 min. Volatiles were identified by comparison of retention times and mass spectra with those of synthetic compounds, with computer data in the NBS75K library, and with our own KEM-EKOL library.

Coupled Gas Chromatographic–Electroantennographic Detection (GC-EAD) Analysis. Four microliters of leaf aeration June samples (*B. pendula* or *B. pubescens*) were injected splitless into a HP 5890 GC equipped with a HP-Innowax column (30 m × 0.25 mm × 0.25 μm) and a 1 : 1 effuent splitter that

allowed simultaneous flame ionization detection (FID) and EAD of the separated volatile compounds (Gries, 1995). Hydrogen was used as carrier gas, and the injector temperature was 225°C. The column temperature was 40°C for 2 min rising to 230°C at 10°C/min. The outlet for the EAD was held in a humidified airstream flowing at 0.5 m/sec over an *Ips typographus* antennal preparation. A glass capillary indifferent electrode filled with Beadle-Ephrussi Ringer and grounded via an Ag–AgCl wire was inserted into the severed beetle's head with the antennae. A similar recording electrode connected to a high-impedance DC amplifier with automatic baseline drift compensation was placed in contact with the distal end of the antennal club.

Adult *I. typographus* for analysis were obtained from continuous cultures maintained on Norway spruce (Schlyter and Anderbrant, 1993). They were taken from generation 94 (T94) of a strain originally collected from Lardal, Norway in 1983, or from a second generation (WW2) of a strain collected in Torsby in 1997. The antennal responses of both sexes (Schlyter and Cederhom, 1981) was measured.

Quantification and Statistical Analysis. For GC-MS, relative amounts of individual compounds were calculated as percentages of whole blends. Absolute amounts of selected compounds were obtained by comparison to the internal standard or the stabilizer. Means were compared by either t tests (two-tailed) or one-way ANOVA of data transformed by log $(X + 1)$, followed by the Ryan, Einot, Gabriel, Welsh (REGW) multiple Q test (SPSS 8.0; Day and Quinn, 1989). In all cases, $\alpha = 0.05$.

RESULTS

Leaf Volatiles and Seasonal Variation

In no case did GC-MSD analyses of ambient air reveal any plant volatiles at detectable levels.

Betula pendula. Monoterpene hydrocarbons, aliphatics (GLVs), and sesquiterpenes were identified from both June and August samples (Table 3). GLVs comprised ca. 45% of the June and 22% of the August samples. The main components in June samples, (Z)-3-hexenyl acetate ($Z3C_6Ac$), (Z)-3-hexen-1-ol ($Z3C_6OH$), (*E*)-β-ocimene, bourbonene, methyl salicylate, α-cubebene, and caryophellene, were also found in similar proportions in August, but at significantly lower levels. The sums of all volatiles also differed between seasons (Figure 2). Some monoterpenes (β-pinene, sabinene), GLVs (hexanal, 1-hexanol), and sesquiterpenes (α-cubebene, bourbonene, and β-cubebene) were present in June samples in relatively large amounts, but were not detected in August. In contrast, (Z)-β-ocimene (5%), butanoic acid (1.9%) and pentanoic acid (34%) were found in August, but not in June. The emission of individual GLVs significantly declined from June to August ($P < 0.05$; t test; Table 3).

TABLE 3. HEADSPACE VOLATILES COLLECTED IN SITU FROM LEAF-BEARING BRANCHES OF FOUR DECIDUOUS TREE SPECIES IN JUNE AND AUGUST (ng/g DRY LEAF WEIGHT), 1997, SWEDEN

Compound	*B. pendula* (Mean ± SD)		*B. pubescens* (Mean ± SD)		*P. tremula* (Mean ± SD)		*S. nigra* (Mean ± SD)	
	June ($N = 6$)	August ($N = 4$)	June ($N = 5$)	August ($N = 4$)	June ($N = 6$)	August ($N = 4$)	June ($N = 6$)	August ($N = 4$)
1. Aliphatic								
Tridecene	5.8 ± 2.3							
1-Hexanol	3.5 ± 4.0		18.2 ± 4.9		0.5 ± 1.1			
(*Z*)-3-Hexen-1-ol	55.0 ± 14.6	26.1 ± 20.1	202 ± 68.0	62.7 ± 34.5	8.8 ± 3.8		9.0 ± 15.2	
(*E*)-2-Hexen-1-ol	4.6 ± 1.4	1.3 ± 1.0	4.2 ± 1.8	1.3 ± 1.0	1.5 ± 1.0	0.6 ± 0.5		1.0 ± 0.8
Heptanol	0.7 ± 1.6							
2-Ethyl-1-hexanol		0.7 ± 1.4					3.1 ± 2.4	2.3 ± 1.6
Octanol + (*Z*)-3-hexenyl isopentanoate	3.8 ± 4.3							
Hexanal	4.7 ± 5.3		1.3 ± 3.0					
(*E*)-2-Hexenal	8.6 ± 11.1	0.7 ± 1.4						
Nonanal	5.8 ± 1.5		11.9 ± 1.2		5.0 ± 1.4		2.0 ± 1.9	
Decanal							10.1 ± 5.4	9.2 ± 7.7
Hexyl acetate	14.4 ± 4.0	2.3 ± 4.5	47.4 ± 13.6	13.9 ± 6.7				
(*Z*)-3-Hexenyl acetate	130 ± 42.8	44.4 ± 40.0	501 ± 246	26.4 ± 16.3	67.6 ± 18.6		14.3 ± 6.0	
(*E*)-2-Hexenyl acetate	4.0 ± 2.1	0.4 ± 0.8	38.7 ± 22.2					
(*Z*)-3-Hexenyl butanoate				20.8 ± 10.1	0.7 ± 1.1			
(*E*)-3-Hexenyl butanoate	7.3 ± 2.1		33.8 ± 11.3	7.3 ± 8.5	4.8 ± 1.4			
3-Hexenyl hexanoate (*E*/*Z*?)					2.9 ± 2.3			
Hexanoate	2.3 ± 3.7	2.6 ± 3.9						
Isopropyl pentanoate	1.8 ± 2.0	3.8 ± 3.4						
2-Pentyl furan	1.9 ± 2.9							
Butanoic acid		7.0 ± 7.6		4.3 ± 8.6		2.0 ± 2.4		
Pentanoic acid		104 ± 62.3						
Hexanoic acid							2.0 ± 3.0	1.8 ± 2.1
Unidentified (bp: 69, 41)					0.4 ± 1.0	3.5 ± 4.0		
Unidentified furanone	5.4 ± 2.6	5.4 ± 6.3						
Unidentified (bp = 71)							1.1 ± 1.8	1.5 ± 1.8
Σ	260	198	858	137	92.2	6.1	41.5	15.8

2. Terpenic								
A. Hydrocarbons								
Monoterpenes								
α-Pinene	11.6 ± 3.0	2.7 ± 3.8	0.9 ± 1.9	186.8 ± 58.5	58.5 ± 65.8	0.5 ± 1.0		
β-Pinene	4.6 ± 4.3		0.8 ± 1.8	102.1 ± 32.1	43.4 ± 43.7			
Sabinene	1.5 ± 3.7		9.5 ± 10.9	1250 ± 366	11.8 ± 8.1	0.4 ± 0.9		
3-Carene	1.9 ± 3.4	0.5 ± 0.9	5.7 ± 6.3	46.4 ± 13.9	76.7 ± 80.3			
2-Carene				31.2 ± 9.2				
Unidentified	1.0 ± 2.6				3.5 ± 3.9			
Limonene					15.6 ± 16.6			
β-Phellandrene + C_{12}	7.7 ± 1.8	2.5 ± 3.3			22.7 ± 20.1			
(*Z*)-β-Ocimene					6.1 ± 4.7		21.2 ± 21.5	
(*E*)-β-Ocimene	22.5 ± 7.0	47.9 ± 41.6	114.8 ± 126.2		120 ± 96.5	4.9 ± 7.9		
p-Cymene				29.5 ± 7.9	0.7 ± 1.1			
Unidentified (m/z 69, 41; MW = 150) (+ tridecane)	13.8 ± 5.9	11.3 ± 2.9	60.7 ± 29.0	13.5 ± 10.2	34.6 ± 16.3		2.6 ± 4.2	5.6 ± 1.3
$\sum$	64.7	64.9	192	1660	394	5.8	23.8	5.6
Sesquiterpenes								
Unidentified terpene (MW = 178)			93.9 ± 35.2	6.6 ± 7.6				
Unidentified terpene (MW = 176)			125.0 ± 35.4					
α-Cubebene			9.7 ± 1.9		3.1 ± 2.6			
Unidentified			47.5 ± 5.8	8.7 ± 5.9				
α-Copaene	22.0 ± 19.1		153.6 ± 50.8	11.9 ± 9.8	17.2 ± 9.0			
Unidentified (bourbonene-alike)	2.2 ± 3.4							
Unidentified				52.8 ± 11.7				
Unidentified terpene (bp = 190)			9.1 ± 5.7					
β-Bourbonene	28.5 ± 39.3		19.9 ± 12.1		5.2 ± 2.0		0.8 ± 1.4	
Unidentified (bp = 161)			14.3 ± 5.4				1.0 ± 2.4	
Unidentified (m/z = 93,161)	1.6 ± 2.5	0.5 ± 1.1			7.2 ± 7.4		1.6 ± 1.9	
Unidentified (bp = 120)	1.5 ± 3.7	0.3 ± 0.7	9.8 ± 4.1		0.9 ± 2.1		3.3 ± 1.8	
Unidentified (bp = 161, 97)	2.1 ± 5.1		12.6 ± 4.4		2.3 ± 2.6		3.3 ± 5.6	
β-Caryophyllene	18.3 ± 3.2	8.6 ± 5.6	496 ± 170	174 ± 44.6	56.0 ± 17.2		1.3 ± 2.0	
allo-Aromadendrene + unidentified (bp = 161)	5.5 ± 6.1	0.9 ± 1.7	38.3 ± 16.1	16.1 ± 0.7	7.5 ± 4.4	0.6 ± 1.2		
α-Humulene			74.1 ± 24.3	19.9 ± 6.5	11.8 ± 4.1	1.6 ± 2.1		
Muurolene	2.8 ± 4.7	17.1 ± 9.8	12.7 ± 3.8		8.0 ± 6.2			
β-Cubene	7.7 ± 2.3		63.1 ± 53.3		11.7 ± 5.2			
Unidentified (bp = 91, 118)			25.6 ± 11.4		8.0 ± 6.2			
Unidentified (bp = 43 > m/z 105,161)	3.3 ± 3.9		13.7 ± 5.7		11.7 ± 5.2		2.6 ± 3.4	2.6 ± 2.3
Unidentified (bp = 121) resp. (bp = 169)			11.6 ± 6.0					

TABLE 3. CONTINUED

Compound	*B. pendula* (Mean ± SD)		*B. pubescens* (Mean ± SD)		*P. tremula* (Mean ± SD)		*S. nigra* (Mean ± SD)	
	June (N = 6)	August (N = 4)	June (N = 5)	August (N = 4)	June (N = 6)	August (N = 4)	June (N = 6)	August (N = 4)
α-farnesene	5.6 ± 3.6	6.8 ± 4.9	47.4 ± 27.9		36.5 ± 24.7	1.5 ± 1.9	10.3 ± 15.3	2.1 ± 1.6
δ-Cadinene (+ unidentified; bp = 161)	5.2 ± 8.2		19.5 ± 6.5					
α-Curcumene		0.8 ± 1.5			194 ± 151.7	0.8 ± 1.6	1.4 ± 2.2	1.7 ± 2.2
Unidentified (bp = 105)					25.7 ± 14.7	1.5 ± 1.8	7.7 ± 11.9	3.8 ± 2.9
Unidentified (MW = 206)			737 ± 332	345 ± 97.9				
Unidentified (bp = 159; MW = 202)	3.8 ± 1.0	0.3 ± 0.6	10.7 ± 3.3		0.8 ± 1.3			
Unidentified terpene (m/z 95, 123, 105)			17.8 ± 3.4					
Unidentified terpene (m/z 95, 105, 161, 176)			12.2 ± 1.8		4.3 ± 3.7			
Unidentified terpene (C_{13}-terpenoid ?)			31.0 ± 8.3					
Unidentified			33.1 ± 7.3					
Unidentified (bp = 161)					0.7 ± 1.1			
Unidentified terpene			5 ± 7.1					
Unidentified			28 ± 10.5	23.1 ± 4.4				
Unidentified (m/z 80, 134, 202)			16 ± 13					
Σ			2190	658	393	6.0	33.2	10.3
B. Oxygenated terpenes								
Hemiterpene								
3-Methyl-1-butanol				31.1 ± 5.1				
Σ				31.1				
Monoterpenes								
(*Z*)-Linaloloxide			7.4 ± 4.5					
Linalool	10.0 ± 3.9	0.5 ± 1.0	51.6 ± 18.7	17.7 ± 1.8	15.3 ± 7.7		9.5 ± 11.5	
Terpinen-4-ol (+ unidentified sesquiterpene)	21.4 ± 16.2							
Geranyl acetone	2.5 ± 3.2				1.8 ± 1.6			
Unidentified terpene (bp = 108, 111; MW = 176)	13.4 ± 7.4			23.2 ± 15.8	5.7 ± 2.6			
Σ	47.3	0.5	59.0	40.9	22.8		9.5	

Sesquiterpenes								
Unidentified alcohol (m/z 96, 79, 109, 123, 138, 207)			20.2 ± 4.7		0.4 ± 1.1			
Caryophyllene oxide			184 ± 50.6	98.9 ± 27.3				
Unidentified terpene (C_{14}-terpene; MW = 190)			33.1 ± 9.9					
Unidentified alcohol			16.9 ± 10.1	10.2 ± 7.2				
Unidentified alcohol (m/z 133, 202, 205, 220)			34.3 ± 33.5	17.5 ± 3.7				
Unidentified alcohol (m/z 109, 79, 202 205, 220)			49.9 ± 19.8	10.0 ± 12.9				
Unidentified alcohol (m/z 136, 202, 205)			33.9 ± 11.3					
Unidentified alcohol (m/z 69, 109, 202 205, 220)			186 ± 111	48.9 ± 20.7				
Unidentified alcohol (m/z 91, 79, 105 202, 205)			73.7 ± 18.7	30.1 ± 9.6				
Unidentified			7.3 ± 5.0					
Unidentified			17.6 ± 7.1					
Unidentified			6.2 ± 4.2					
Unidentified			42.1 ± 11.1	14.3 ± 9.9				
Unidentified			9.9 ± 6.9					
Unidentified (or C_{16}-terpene ?)			15.1 ± 14.1					
Unidentified (m/z 91, 105, 131, 159, 187 202, 220)			784 ± 417	266 ± 94.7				
Unidentified (m/z 136, 91, 105, 205, 220)			114.0 ± 37.9	47.3 ± 34.4				
Unidentified (m/z 109, 105, 151, 205, 220)			31.1 ± 13.9					
$\sum$			1660	543	0.4			
Aromatic								
Toluene	13.4 ± 3.0	6.6 ± 4.6	0.9 ± 2.1		3.4 ± 8.2	5.6 ± 1.1	8.3 ± 2.1.	5.2 ± 1.1
Styrene					7.1 ± 17.3			
Benzyl alcohol					0.3 ± 0.7			
Phenol								
Benzaldehyde		0.5 ± 1.1			3.0 ± 2.7		0.9 ± 1.6	
Methyl benzoate	6.9 ± 6.2	3.1 ± 4.4						
Ethyl benzoate	2.6 ± 4.6	2.2 ± 4.4						
Methyl salicylate	26.3 ± 22.0	1.2 ± 1.7	9.2 ± 11.8		14.9 ± 3.8		0.7 ± 1.7	
Jasmone (Z/E?)			13.2 ± 4.6					
$\sum$	49.2	13.6	23.4	0.0	28.6	5.6	9.9	5.2
Sum of all compounds	531 ± 117	312 ± 93	4980 ± 1690	3070 ± 843	931 ± 281	24 ± 7.0	118 ± 69	37 ± 18.9

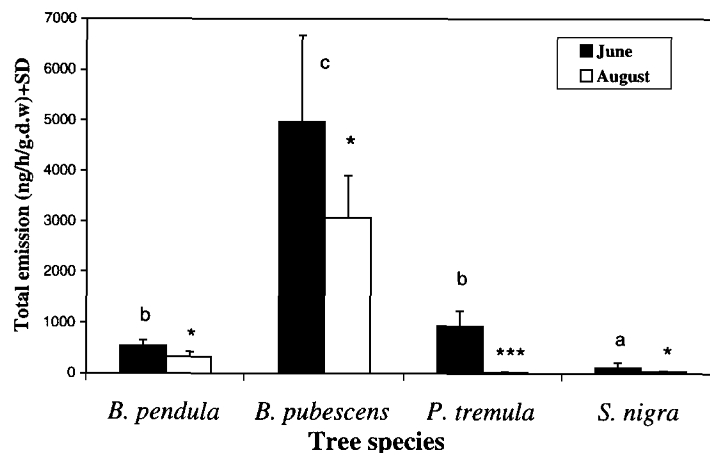

FIG. 2. Seasonal changes in total volatile emissions from in situ leaves of four deciduous tree species. Differences of mean total emission rates between June ($N = 5$–6) and August ($N = 4$) samples for each tree species compared with *t*-tests (two-tailed) indicated by $^{*}P < 0.05$; $^{**}P < 0.01$, and $^{***}P < 0.001$ levels. Bars for June samples with different letters are significantly different, ANOVA followed by REGW Q tests, $P < 0.05$.

Betula pubescens. Unlike *B. pendula*, *B. pubescens* emitted considerable numbers of sesquiterpene alcohols and other oxides at high levels in both June (35%) and August (18%) samples (Table 3). The most abundant components in June were (*E*)-β-ocimene, (Z)-3-hexenyl acetate, (Z)-3-hexen-1-ol, α-copaene, β-caryophyllene, an unidentified sesquiterpene (MW = 206), caryophyllene oxide, and two unidentified sesquiterpene oxides (m/z 91, 105, 131, 159, 187, 202, 220; and m/z 69, 109, 202, 205, 220). GLVs made up only ca. 17% and 3.6% of the blends in June and August, respectively. Both the total volatiles (Figure 2) and amounts of the dominant components (Table 3), including two major GLVs, (Z)-3-hexenyl acetate, and (Z)-3-hexen-1-ol, declined significantly in August. Some GLVs (hexanal and 1-hexanol), sesquiterpenes, and sesquiterpene oxides were detected in June, but not in August. However, monoterpenes present in June as minor components (some of them in trace amounts) became the major constituents (53%) in August, with sabinene being the most dominant component at 40.6% (125 times higher than in June).

Populus tremula. Similar to *B. pendula*, mainly monoterpenes, GLVs and sesquiterpenes were found from June samples of *P. tremula*, with monoterpenes being the most dominant (37%) (Table 3). GLVs including (Z)-3-hexenyl acetate, (Z)-3-hexen-1-ol, 1-hexanol, and (*E*)-2-hexen-1-ol (*E*2C_6OH), made up only 8.8% of the total blend in June and were not detected in August except for a trace of (*E*)-2-hexen-ol. The major components in summer were (*E*)-β-ocimene,

3-carene, α-pinene, (Z)-3-hexenyl acetate, (Z)-3-hexen-1-ol, α-farnesene, and β-caryophyllene. The total amount of volatiles in August was significantly lower than in June (Figure 2). Only a few compounds were detected in August, again with monoterpenes as the major constituents (42%). However, butanoic acid was found only in August as 9.6% of the blend.

Sambucus nigra. Compared to the other species, *S. nigra* emitted volatiles at significantly lower levels in both June and August (Figure 2). The major components were (*E*)-β-ocimene, (Z)-3-hexenyl acetate, (Z)-3-hexen-1-ol, an unidentified sesquiterpene (bp = m/z 43 > 105, 119, 161), α-farnesene, and β-caryophyllene. GLVs made up 14% of total volatiles in June. In August, most of components found in June were absent (Table 3).

Effects of Temperature on Emission of Leaf Volatiles

The number and the total amount of volatiles released from *B. pendula* and *S. nigra* were higher at 32°C than at 16°C (Figures 3 and 4). For *B. pendula*, the only compounds that were not captured at significantly different levels at the two temperatures were hexanal, (*E*)-2-hexenal, α-pinene, sabinene, and β-cubebene (Figure 3). For *S. nigra*, the total emission of volatiles, as well as the (Z)-3-hexen-1-ol, (Z)-3-hexenyl acetate, and β-caryophyllene increased significantly from 16°C to 24°C ($P < 0.05$, ANOVA), after which the release rate plateaued (Figure 4). The release rate of a few minor components, e.g., toluene, 3-carene, nonanal, and decanal, did not change with temperature.

GC-EAD Analysis

Three GLVs, (Z)-3-hexen-1-ol, 1-hexanol, and (*E*)-2-hexen-1-ol, from both *B. pendula* and *B. pubescens* leaves consistently elicited strong responses from five *I. typographus* antennae (Figure 4). The amounts of these compounds from *B. pendula* and *B. pubescens* samples directed toward the antenna were estimated as: 2.33 and 28 ng, 0.43 and 2.97 ng, and 0.40 and 0.71 ng, respectively. Two other compounds from *B. pendula*, linalool and geranyl acetone, were antennally active in only one GC-EAD run. No responses were detected to any other compounds. No differences in EAD responses were found between males ($N = 3$) and females ($N = 2$).

DISCUSSION

Leaf Volatiles and Seasonal Variation. The absence of plant volatiles in ambient air samples indicates that volatiles found in the enclosure samples were emitted from the plants. Although monoterpenes, aliphatics, and sesquiterpenes were produced by all four species, there were pronounced interspecific differences. In particular, *B. pendula* and *B. pubescens* are sympatric and morphologi-

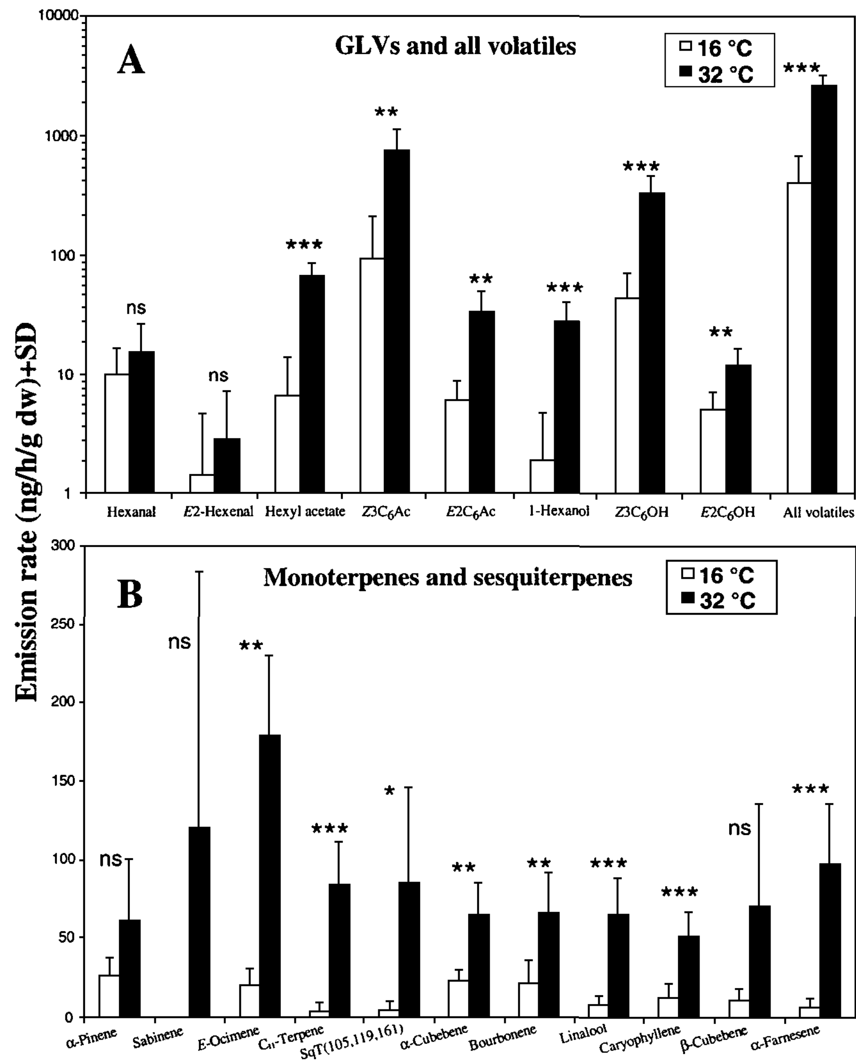

FIG. 3. Effects of temperature on volatile emission from *B. pendula* leaves sampled ex situ. (A) Total volatile and GLVs emissions; (B) major monoterpenes and sesquiterpenes. Differences in mean emissions between 16°C (N = 5) and 32°C (N = 5) compared with t tests (two-tailed) indicated by $^{*}P < 0.05$; $^{**}P < 0.01$, and $^{***}P < 0.001$ levels.

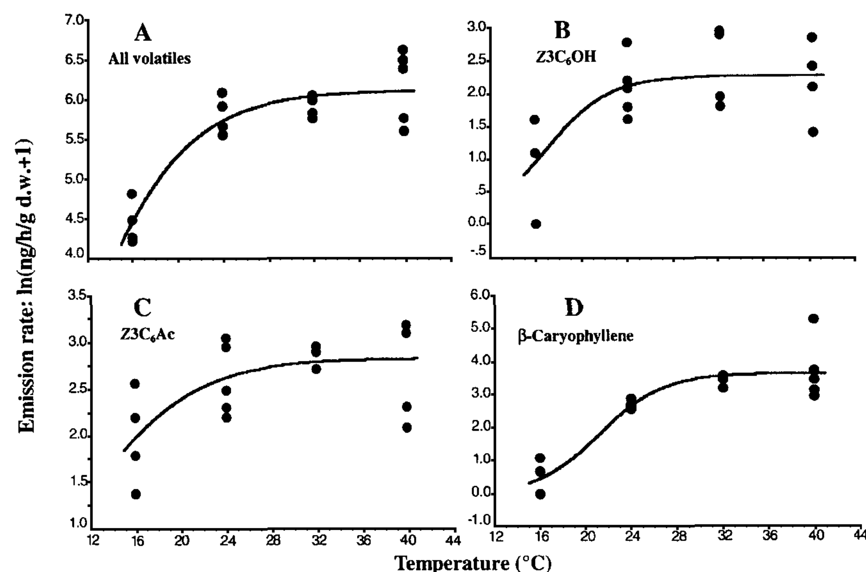

FIG. 4. Relationship between temperature (x) and volatile emissions (y) from *S. nigra* leaves sampled ex situ fitted by Verhulst nonlinear regression: $y = \ln$ (emission rate + 1) $= b_1/[1 + b_3 * \exp(-b_2 * X)]$. (A) All volatiles: $y = 6.1/[1 + 11.9 * \exp(-0.21x)]$, $r^2 = 0.84$; (B) (Z)-3-hexen-1-ol: $y = 2.3/(1 + 480 * \exp(-0.37 * x)]$, $r^2 = 0.52$; (C) (Z)-3-hexenyl acetate: $y = 3.9/(1 + 13 * \exp(-0.22 * x)$, $r^2 = 0.43$; (D) β-caryophyllene: $y = 3.6/(1 + 2300 * \exp(-0.36 * x)$, $r^2 = 0.86$.

cally and ecologically similar in Scandinavia, but the compositions of their leaf volatiles, especially the occurrence of sesquiterpene alcohols and other oxides in the latter species, are strikingly different. It is not clear that the differences have a genetic basis. However, *B. pendula* has a diploid chromosome number ($2n = 28$), while *B. pubescens* has a polyploid chromosome number ($2n = 56$) (Inki and Väisänen, 1980). In June, *B. pubescens* released 5, 10, and 100 times more volatiles than *P. tremula*, *B. pendula*, and *S. nigra*, respectively (Figure 2). Similar volatile profiles and emission rates were also found in June 1998 samples from *B. pendula*, *B. pubescens*, and *P. tremula* (4 samples/species) in Asa, ca. 250 km NE of the 1997 sampling sites (Zhang et al., unpublished data).

The predominance of GLVs released from *B. pendula* leaves is in agreement with the results of Isidorov et al. (1985) and König et al. (1995). König et al. (1995) found similar release rates of (Z)-3-hexenyl acetate and (Z)-3-hexen-1-ol as in our study, but neither Isidorov et al. (1985) nor König et al. (1995) detected the release of any other GLVs. They did detect monoterpenes and sesquiterpenes but with less diversity than in our samples.

Conifers exhibit a strong seasonal variation in both composition and amount of needle volatiles, mainly monoterpenes (von Rudloff, 1972; Hrutfiord et al., 1974; Schönwitz et al., 1990), depending on temperature (Tingey et al., 1980; Yukouchi and Ambe, 1984), light intensity (Yukouchi and Ambe, 1984), and phytogenic effects like foliage drop and blooming (Arey et al., 1991). Moreover, the conifer volatile spectrum may increase in richness in current-year foliage as the season progresses (Brooks et al., 1987). In contrast, all four angiosperm species tested here had significantly lower numbers and amounts of volatiles emitted in August than in June (Figure 2, Table 3). This variation may be due to maturation of leaves because temperature and light intensity during the aerations in both June and August were almost the same (Table 1). The higher concentration of volatiles released in the early summer might be an adaptive trait associated with deterrence of phytophagous insects.

Temperature Effects. Temperature is one of many factors believed to influence the volatile emission of plants (Charron et al., 1995). Our ex situ study exposed cut branches to temperature ranges wide enough to cover natural conditions in early and mid-summer (Table 2) and revealed significant increases in the release of monoterpenes, GLVs, and sesquiterpenes from *B. pendula* from 16°C to 32°C, the two temperatures tested (Figure 3). In *S. nigra*, the increase in release of all the above classes of compounds occurred between 16°C and 24°C, with only a slight increase above 24°C, a trend described well by Verhulst nonlinear regression curves (Figure 4). The absence of pronounced increases in emissions at high temperature levels might be due to the defense system of the plant, including closing the leaf stomata.

It was reported that monoterpene emissions increased exponentially with temperature from leaves of *Eucalyptus globulus* L. (Guenther et al., 1991), *Quercus ilex* L. (Staudt and Seufert, 1995), and from foliage of many conifer species, with little shift in the relative proportions of individual monoterpenes (Dement et al., 1975; Kamiyama et al., 1978; Tingey et al., 1980, 1991; Yukouchi and Ambe, 1984; Lamb et al., 1985; Juuti et al., 1990). These emission rates increase more rapidly than do comparable changes in vapor pressure and may be explained by alterations of the pathway conductance as well as the change in vapor pressure (Tingey et al., 1991). Almost no data are available on the effects of temperature on GLV and sesquiterpene emissions (Guenther et al., 1991; Winer et al., 1992).

Electrophysiological Activity. Our results clearly showed for the first time that three GLV alcohols, (*Z*)-3-hexen-1-ol, 1-hexanol, and (*E*)-2-hexen-1-ol, emitted from both *B. pendula* and *B. pubescens* intact leaves induced a very strong EAD response by *I. typographus* (Figure 5). In one GC-EAD run, linalool and geranyl acetone from *B. pendula* also produced slight responses. Other GLVs from the nonhost leaves, including the most dominant GLV, (*Z*)-3-hexenyl acetate, did not elicit any EAD response. These three antennally active

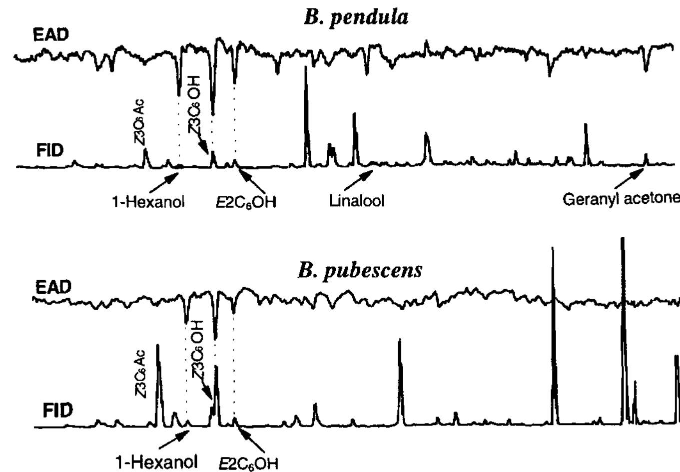

FIG. 5. Simultaneously recorded flame ionization detector (FID) and electroantennographic detector (EAD) responses using antennae of *Ips typographus* in response to leaf volatiles of *B. pendula* (upper trace) and *B. pubescens* (lower trace). No difference in GC-EAD responses between males ($N = 3$) and females ($N = 2$) to the leaf volatiles.

GLVs were identified as well from *P. tremula*, *S. nigra* (Table 3), and *S. racemosa* (Zhang, unpublished data). 1-Hexanol was also detected in trace amounts from the female flowers of *Picea abies* and *Pinus sylvestris*, but not from twigs with needles (Borg-Karson et al., 1985). Our further GC-MS analyses did not find any of these antennally active GLVs at detectable levels from aeartion samples of fresh bark and branches with needles of host tree *Picea abies* (Zhang et al., unpublished data). A blend of five GLVs, including the three active alcohols above, and linalool, had pronounced inhibitory effects on the efficiency of attractant-baited traps to *I. typographus*, *I. duplicatus*, and *T. piniperda* (Schlyter et al., 1995).

Tømmerås (1989) and Tømmerås and Mustaparta (1989) found some receptor neurons from *I. typographus* and *T. lineatum* antennae, that responded to unknown birch bark volatiles. In agreement with our results, Wilson et al. (1996) found that *Dendroctonus ponderosae* antennae responded in GC-EAD analysis to 30-ng doses of all five green leaf alcohols tested [1-hexanol, (*E*)-2-hexen-1-ol, (Z)-2-hexen-1-ol, (*E*)-3-hexen-1-ol and (Z)-3-hexen-1-ol], but not to the aldehydes, hexanal, or (*E*)-2-hexenal, or to alcohol or aldehyde homologs with more or fewer than six carbon atoms.

GLVs are produced by plants as a product of oxidation of leaf surface lipids. They have been assigned several roles in insect behavior, including: (1) host plant finding by phytophagous insects (Visser, 1986); (2) enhancement of attrac-

tion to insect pheromones (Dickens et al., 1990); (3) host finding by parasitoids of lepidopterous larvae (Whitman and Eller, 1990); and (4) interruption of aggregation pheromone response in conifer bark and ambrosia beetles, as reviewed by Deglow and Borden (1998a). Hexanal and hexan-1-ol disrupted attraction of *D. frontalis* Zimm, to traps baited with attractant semiochemical, and hexanal had a similar effect on *Ips grandicollis* (Eichhoff) and *I. avulsus* (Eichhoff) (Dickens et al., 1992). A blend of four green leaf alcohols interrupted attraction by *D. ponderosae* Hopkins, to pheromone-baited traps, whereas a blend of two green leaf aldehydes was inactive (Wilson et al., 1996). Similar to *D. frontalis*, hexanal, (*E*)-2-hexenal, and 1-hexanol were disruptive in *D. rufipennis* and (*E*)-2-hexenal and two GLV alcohols, (Z)-2-hexen-1-ol and (*E*)-2-hexen-1-ol significantly reduced the numbers of *D. brevicomis* captured (Poland et al., 1998). GLV alcohols have also been shown in trapping experiments to disrupt the response to aggregation pheromones by conifer-infesting ambrosia beetles, including *T. lineatum* (Borden et al., 1997), *G. sulcatus*, and *G. retusus* (Deglow and Borden, 1998a,b). European field experiments with 1-hexanol and (*Z*)-3-hexen-1-ol tested singly (Byers et al., 1998) and further a GC-EAD study with a nine-component synthetic GLV mixture containing two aldehydes, one acetate, and six alcohols and a walking bioassay as well as field trapping tests that showed repellency only by the alcohols (Zhang et al., in preparation), support the results of Wilson et al. (1996) and corroborate our GC-EAD findings.

CONCLUSION

We have shown that angiosperm trees, commonly existing in mixed forests, emit GLVs, monoterpenes, and sesquiterpenes in early and mid-summer when *I. typographus* in flight are searching for hosts. The significant EAD activity of the green leaf alcohols emitted from nonhost trees suggests that they may direct host-seeking *I. typographus* away from habitats predominating in nonhost trees, e.g., *B. pendula*, *B. pubescens*, and *P. tremula*. Leaf odors may also help *Ips* and *Tomicus* spp. to detect and fly above a deciduous understory, e.g., *S. nigra* and *S. racemosa*. As postulated by several authors (Borden et al., 1998, Byers et al., 1998; Schlyter and Birgersson, 1999), bark beetles may have evolved these behaviors to avoid the dangers of prolonged dispersal associated with investigating habitats with nonhosts, or even nonhost trees themselves.

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EVOLUTION AND ADAPTIVE SIGNIFICANCE OF LARVAL MIDGUT ALKALINIZATION IN THE INSECT SUPERORDER MECOPTERIDA

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Abstract—The phylogenetic distribution of reported midgut pH values among larval Mecopterida supports a model in which the common ancestor of this group possessed an alkaline midgut, with subsequent loss of this trait in the lineage leading to the muscomorphan Diptera. The relationship between midgut pH and diet guild rank within the Lepidoptera and Diptera was tested by assigning numerical values to diet guilds (i.e., fruit, grasses, herbs, trees and shrubs, and organic detritus). Lepidopteran superfamilies were found to differ significantly in both midgut pH and in diet guild rank. Regression of mean superfamily midgut pH against mean superfamily diet guild rank yielded an R^2 of 0.79 (N = 10), whereas regression of species midgut pH against species diet guild rank yielded an R^2 of only 0.15 (N = 60). Species feeding on foliage of plant taxa high in tannins and on Solanaceae have midgut pH values above 9, and midgut pH in species feeding on these taxa is positively related to diet guild. In contrast, species feeding on the foliage of plant taxa containing terpenes, DIMBOA, glucosinolates, and pyrrolizidine alkaloids have midgut pH values near 8, and midgut pH of these species is either not related to diet guild (all species) or is negatively related to diet guild rank when the analysis is limited to the Noctuoidea. The data suggest that decreased midgut pH in species feeding on plants containing terpenes, DIMBOA, glucosinolates, and pyrrolizidine alkaloids may be an adaptive response that overrides selection for high pH in the presence of tannins and that midgut pH may be one factor contributing to the limitation of the host plant range of many species of lepidopteran herbivores.

Key Words—Insect–plant interaction, midgut alkalinization, phylogenetic distribution, Mecopterida, Diptera, Lepidoptera, Trichoptera, polyphenolics, alkylating agents.

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INTRODUCTION

Larvae of a number of endopterygote insects, including at least some members of each of the orders Coleoptera, Diptera, Trichoptera, and Lepidoptera, have a highly alkaline region within their midguts (Bayon, 1980; Dadd, 1975; Martin et al., 1980; Espinoza-Fuentes et al., 1984; Stiles and Paschke, 1980; Lacey and Federici, 1979; Undeen, 1979; Waterhouse, 1949; Shinoda, 1930). In contrast, the majority of exopterygote insects (hemimetabolous insects such as Hemiptera and Orthoptera) have midguts that are near neutral or mildly acidic (Terra and Ferreira, 1994). The midgut pH of an individual insect does not appear to be regulated in response to changes in diet. Instead, each species appears to maintain a set midgut pH (Johnson and Felton, 1996; Cooper and Vulcano, 1997). Indeed, *Manduca sexta* maintained for generations on artificial diet consisting mainly of wheat germ have midgut pH values well above 10 (T. M. Clark, unpublished observations). The evolution in a species of a specific midgut pH setpoint is not unexpected, as digestive enzymes have evolved specific pH optima that must match the gut pH for maximum efficiency (Applebaum, 1985).

The relative importance of phylogeny and adaptation in explaining the distribution of midgut pH among insects is presently unclear. Waterhouse (1949) and Terra et al. (1996) concluded that midgut pH is more closely correlated with phylogenetic position than with diet. Others have argued for adaptive responses of midgut pH to diet and have proposed that the high midgut pH of some lepidopteran larvae aids in the extraction of protein from insoluble complexes with tannins (Berenbaum, 1980) or phenolics (Felton and Duffey, 1991) or in the extraction of hemicelluloses (Terra, 1988). The acidic midgut of the Colorado potato beetle has also been interpreted as an adaptation to avoid the antinutritive effects of phenolics (Felton et al., 1992). No study to date has explicitly investigated the relative contributions of phylogeny and diet in explaining midgut pH within an insect lineage. In the present analysis, I have inferred the evolutionary origin of larval midgut alkalinization within the Mecopterida and have also investigated the relationship between midgut pH, insect phylogeny, and chemical characteristics of diet in order to infer the adaptive significance of this trait among larval Lepidoptera and Diptera.

METHODS AND MATERIALS

Phylogenetic Distribution of Midgut Alkalinization Within the Mecopterida. Midgut pH values were obtained from the literature [Lepidoptera (Berenbaum, 1980; Waterhouse, 1949) Trichoptera (Shinoda, 1930; Martin et al., 1981) Diptera (refs. in Table 1)]. Phylogenies of the Mecopterida were obtained from Hennig (1981), Kristensen (1991), and Whiting et al. (1997). Although these

TABLE 1. MIDGUT pH OF DIPTERAN LARVAE

Family	Infraorder	Midgut pH	Reference
Tipulidae	Tipulomorpha	11.2	Martin et al. (1980)
Culicidae	Culicomorpha	>9.6–>10	Dadd (1975), Stiles and Paschke (1980)
Simuliidae	Culicomorpha	>9.8–11.4	Undeen (1979), Lacey and Federici (1979)
Sciaridae	Bibionomorpha	>8.9–10.2	Terra et al. (1979), Espinoza-Fuentes et al. (1984)
Calliphoridae	Muscomorpha	2.8–4.1	Greenberg (1968)
Sarcophagidae	Muscomorpha	3.1–3.3	Greenberg (1968)
Muscidae	Muscomorpha	3.1–3.9	Greenberg (1968), Terra and Regel (1995)
Tephritidae	Muscomorpha	3.4	Lemos and Terra (1991b)

phylogenies differ, the placement of groups with known midgut pH values were identical in all three phylogenies, suggesting that the phylogeny used in this analysis is robust.

Phylogenies of the order Diptera to the level of infraorder were obtained from Hennig (1981), Wood and Borkent (1989), and Woodley (1989). The Diptera are traditionally divided into two suborders, the Nematocera and the Brachycera (Wood and Borkent, 1989; Woodley, 1989). The Nematocera are paraphyletic, as the Brachycera arose from a nematoceran lineage, possibly within the Bibionomorpha (Hennig, 1981). Hennig (1973) divides the Suborder Nematocera into four infraorders, the Tipulomorpha, Psychodomorpha, Culicomorpha, and Bibionomorpha. Wood and Borkent (1989) recognize seven infraorders, including the four described by Hennig (1973). As all available data on nematoceran midgut pH fall into infraorders recognized by both Hennig (1973) and Wood and Borkent (1989), the dipteran phylogeny used in this analysis also appears robust and the further division of this group by Wood and Borkent (1989) is irrelevant in terms of the present analysis. All data on brachyceran midgut pH are from members of the Infraorder Muscomorpha within the cyclorrhaphan Diptera (refs. in Table 1; Woodley, 1989).

Reported Midgut pH Values in Larval Diptera, Lepidoptera, and Trichoptera. Members of dipteran infraorders Tipulomorpha, Culicomorpha, and Bibionomorpha investigated to date have highly alkaline midguts (Table 1), while members of other nematoceran infraorder(s) have not been investigated. In contrast, the Muscomorpha appear to have lost the alkaline anterior region characteristic of Nematocera and instead are characterized by a highly acidic midgut region (Table 1). All Lepidoptera investigated to date have alkaline midguts, including carnivorous species and those that feed on wax or wool (Waterhouse, 1949; Berenbaum, 1980). Reported midgut pH values for Trichoptera range from

slightly to highly alkaline (Shinoda, 1930, Martin et al., 1981), although species reported to have only slightly alkaline midguts were found to have midgut enzyme pH optima near 9 (Martin et al., 1981), suggesting that measured pH values in the midgut may be underestimates of the in vivo values. In any case, the midgut of larval Trichoptera is alkaline (Shinoda 1930; Martin et al., 1981). No midgut pH data are available for other orders within the Mecopterida, and these taxa are therefore omitted from the analysis. Similarly, analysis of midgut pH values of nonmecopteridan endopterygotes is outside the scope of the present study and an exhaustive search of the literature for midgut pH values of these insects has not been performed.

Analysis of Adaptive Significance of Midgut Alkalinization. Midgut pH values and host-plant families for each of 60 species of phytophagous Lepidoptera were obtained from the review of Berenbaum (1980), and those lepidopteran species were assigned to superfamilies according to Nielsen and Common (1991). When pH values were available for a species from more than one report, the mean of the reported values was used in the analyses except when using a nested ANOVA design when mean values from each report were included. The lepidopteran values were obtained by many investigators using a variety of techniques. Midgut pH values of larval Diptera were obtained from the references listed in Table 1. Many of the dipteran values were obtained from in vivo pH indicator studies and are acknowledged in the primary literature to be probable underestimates of the true pH.

Reported diets were assigned to guilds and ranked as follows: fruit; 1, grasses; 2, herbs; 3, shrubs and trees; 4, organic detritus; 5. This ranking considers such factors as protein availability, concentrations and types of plant secondary compounds, and leaf structural toughness (Scriber and Slansky, 1981).

Analyses and Statistics. The phylogenetic distribution of an alkaline larval midgut was revealed by plotting its occurrence on a phylogeny of the Mecopterida. Possible sequences of events that could lead to the observed distribution of this trait among the Mecopterida were then generated. The evolutionary scenario requiring the fewest evolutionary changes to arrive at the present distribution was chosen as the most likely based on the principle of parsimony.

Regressions and single-factor ANOVAs were performed with Excel. The a posteriori tests of means following single-factor ANOVA were performed by Student-Newman-Keuls comparisons of means test (Sokal and Rohlf, 1969). Nested ANOVA of species within superfamilies against midgut pH were performed with SAS. In this case superfamilies represented by a single species were eliminated from the analysis, leaving a total of eight superfamilies.

Extreme generalists (defined for the purposes of this analysis as those feeding on more than five host-plant families) were eliminated from the analysis of the relationship between herbivore midgut pH and plant family. Such species may have evolved different strategies for dealing with allelochemicals and tend

to blur patterns observed among nongeneralists. Host-plant taxa consumed by only a single lepidopteran species were also eliminated. The midgut pH of each of the remaining 44 lepidopteran species was then assigned to each of its host-plant taxa, so that a species feeding on N taxa was represented N times in the data set unless it was the only species feeding on some number, Y, of those N taxa, in which case it was represented $N - Y$ times. Plants listed by Berenbaum (1980) as Coniferae were pooled with those listed as Pinaceae to form a single group (labeled conifers). The a posteriori comparisons of means were performed following ANOVA by using the Student-Newman-Keuls test (Sokal and Rohlf, 1969) in order to determine which means differed significantly at $P < 0.05$. No attempt was made to relate observed differences in herbivore midgut pH among plant taxa to plant phylogeny.

RESULTS

Phylogenetic Distribution of Larval Midgut Alkalinization Within Endopterygota. All members of the Mecopterida, with the exception of the derived dipteran lineage Muscomorpha, possess alkaline midguts. Several evolutionary scenarios could explain this distribution of midgut pH. Most parsimonious is a model in which larvae of the common ancestor of the Diptera and the Lepidoptera/Trichoptera clade possessed an alkaline midgut (Figure 1). This scenario invokes a single evolutionary event, a change from an alkaline to an acidic midgut region in the lineage leading to the Brachycera. A model in which the ancestral Diptera and the ancestral Lepidoptera/Trichoptera acquired this trait independently requires at least three events, while other models require more. It is clear that the acidic region found in the midgut of larval brachyceran Diptera is a derived character that arose following the divergence of this lineage from a nematoceran ancestor.

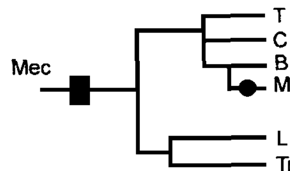

FIG. 1. A phylogeny of the Mecopterida showing the distribution of alkaline and acidic midguts at the level of Infraorder. The hypothetical origin of an alkaline larval midgut is represented by (■), an acidic midgut by (●). Taxa for which no data are available are not represented. T = Tipulomorpha, C = Culicomorpha, B = Bibionomorpha, M = superfamily Muscoidea, L = Lepidoptera, Tr = Trichoptera. Data are from Berenbaum (1980), Shinoda (1930), Martin et al. (1981) and references in Table 1.

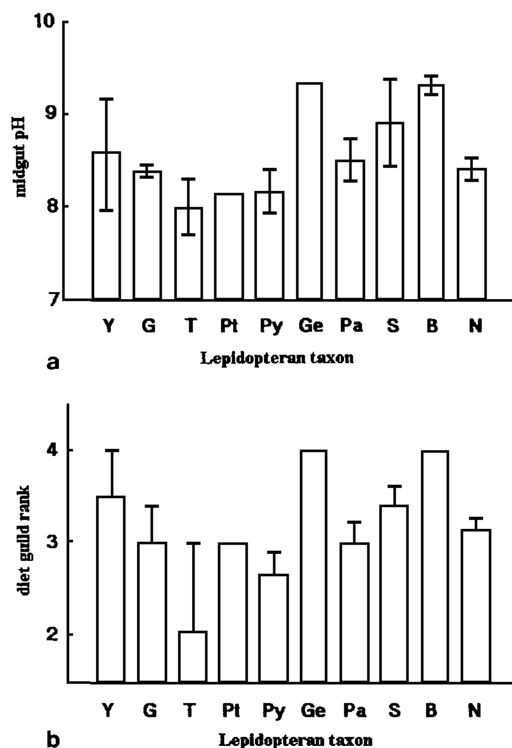

FIG. 2. Significant differences exist among lepidopteran superfamilies in (a) midgut pH ($P < 0.05$, single factor ANOVA) and (b) diet guild ($P < 0.05$, single factor ANOVA). Diet guilds were ranked as fruit = 1, grasses = 2, herbs = 3, trees and shrubs = 4. Y = Yponomeutoidea (2; these numbers are N values—see text), G = Gelechioidea (4), T = Tortricoidea (4), Pt = Pterophoroidea (1), Py = Pyraloidea (9), Ge = Geometroidea (1), Pa = Papilionoidea (7), S = Sphingoidea (4), B = Bombycoidea (4), N = Noctuoidea (24). Y and G are members of the Ditrysia, while the remaining taxa belong to the Apoditrysia. Bars represent SE. Data are from Berenbaum (1980).

Analysis of Larval Midgut Alkalinization Within Lepidoptera. Of the 60 lepidopteran species in the data set of Berenbaum (1980), most (41 species) fed on only a single host-plant taxon, nine species fed on two to five host-plant taxa, and the remaining 10 species fed on more than five host-plant taxa. Of these 10 species, eight fed on 18 or more host-plant taxa.

Midgut pH and diet guild rank differed significantly among lepidopteran superfamilies (Figures 2a and b). Regression of mean superfamily midgut pH against mean superfamily diet guild ranking was highly significant ($R^2 = 0.79$, $P < 0.001$, $y = 0.65x + 6.54$, $N = 10$, data not shown). Despite the strong rela-

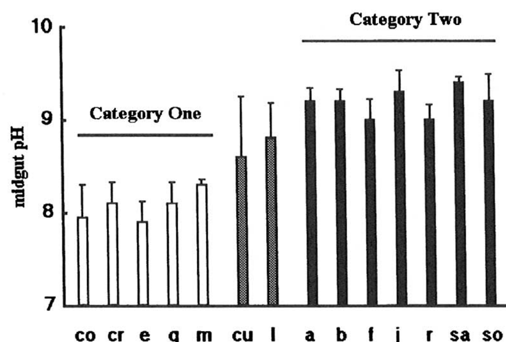

FIG. 3. Midgut pH values of lepidopteran species are correlated with host-plant taxa ($P < 0.05$, single-factor ANOVA). Plant taxa utilized by only one species and lepidopteran species utilizing more than five host-plant families are excluded from the analysis. Taxa represented by white bars (category 1) are significantly different from those represented by black bars (category 2), while no differences exist among white bars or among black bars. Taxa represented by grey bars (cu and l) are not significantly different from either group. Error bars represent the standard errors of the means. co = conifers (2; these numbers are N values—see text), cr = Cruciferae (7), e = Euphorbiaceae (4), g = Graminea (8), m = Malvaceae (4), cu = Cucurbitaceae (2), l = legumes (3), a = Aceraceae (2), b = Betulaceae (2), f = Fagaceae (6), j = Juglandaceae (2), r = Rosaceae (7), sa = Salicaceae (3), so = Solanaceae (3). Data are from Berenbaum (1980).

tionship between these parameters at the level of superfamily, regression of diet guild against midgut pH at the species level yielded $R^2 > 0.15$ ($P > 0.26$, $y = 0.16x + 7.22$, $N = 60$, data not shown). Nested SAS ANOVA of species within superfamilies produced similar results (superfamilies: df; 7, type III SS; 6.68, MS; 0.95, F; 2.47, $P < 0.05$, species: df; 40, type III SS; 15.45, MS; 0.39, F; 0.83, $P > 0.68$). Furthermore, within each of the seven superfamilies that contain more than two data points, in only one instance is an extreme pH matched with an extreme diet rank.

Significant differences were revealed among host-plant taxa in herbivore midgut pH values (Figure 3, $P < 0.0001$, single-factor ANOVA). Student-Newman-Keuls a posteriori multiple comparison tests revealed two major categories of plants in the data set. The mean midgut pH of species utilizing conifers, Euphorbiaceae, Malvaceae, Graminea, and Cruciferae is near 8, and no significant differences occur among these taxa, which will be referred to subsequently as category 1. In contrast, the mean midgut pH values of species consuming Aceraceae, Betulaceae, Fagaceae, Juglandaceae, Rosaceae, Salicaceae, and Solanaceae are above 9 (Figure 3), and again, no significant differences occur among means of these taxa. These taxa will be referred to subsequently as category 2. The mean midgut pH of herbivores feeding on each taxa within

category 1 are significantly different from those feeding on all taxa within category 2, and vice versa (single-factor ANOVA followed by Student-Newman-Keuls a posteriori multiple comparisons test, $\alpha = 0.05$) The midgut pH values of species feeding on Cucurbitaceae or on legumes were not statistically different from those in either category 1 or category 2.

The relationship between midgut pH and diet guild rank was determined separately for species feeding on plants in category 1 and category 2. For the purposes of this analysis, *Crotalaria* (a legume) was included in category 1 because the characteristic pyrrolizidine alkaloids of this group undergo chemical reactions similar to those of category 1 allelochemicals such as terpenes and isothiocyanates (Felton, 1996), as discussed below. The regression between diet guild and midgut pH of species feeding on plant material in category 2 was significant, with greater pH in herbivores feeding on foliage than in those feeding on fruit (Figure 4; $R^2 = 0.42$, $y = 0.38x + 7.70$, $P < 0.05$, $N = 11$). Detritivorous aquatic dipteran larvae feed largely on decaying plant material in which the remaining protein is found in insoluble complexes with polyphenols such as lignins and tannins (Suberkropp et al., 1976). Diptera were therefore also included in this analysis by assigning organic detritus a guild ranking of 5. Inclusion of the detritivorous Diptera did not alter the results of the analysis ($R^2 > 0.47$, $P < 5 \times 10^{-5}$, $N = 24$, not shown). In contrast, the regression between diet guild and pH of all species feeding on plants in category 1 was not significant (Figure 4; $R^2 = 0.006$, $P > 0.69$, $N = 27$). The relationship between diet guild and midgut pH among Noctuoidea was significant if the analysis was limited to species feeding on plants in category 1 (Figure 4; $R^2 = 0.36$, $y = -0.27x + 8.99$, $P < 0.05$, $N = 12$), whereas these parameters were not related among the Noctuoidea as a whole ($R^2 = 0.0005$, not shown). Five lepidopteran superfamilies in the data set contain species that feed on plants in category 1 and species that feed on plants in category 2. In each of these superfamilies, midgut pH of species feeding on plants in category 2 were higher than those of species feeding on plants in category 1, and the midgut pH values of species within each superfamily showed very little overlap between the two categories (Figure 5).

It was noted during the course of this analysis that most moderate generalists (2–10 host-plant taxa) limited their host-plant range to members of a single category (generally category 2). Of particular interest is *Lymantria monacha* (Lymantriidae), which feeds on Pinaceae (category 1) as well as on Rosaceae and Fagaceae (category 2). Significantly, the reported midgut pH of this species is only 8.3, despite the high tannin content of its hostplants (see Discussion below). With the exception of this species, only species categorized as extreme generalists (defined for the purposes of this study as species feeding on more than five plant families) fed on members of both categories of plant taxa revealed in the analysis.

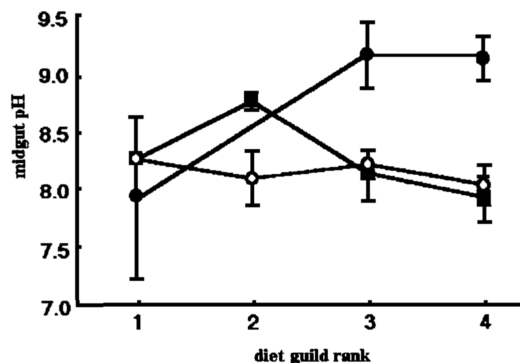

FIG. 4. Midgut pH values of mecopteridan species are related to the chemical composition of the diet. Species of Lepidoptera consuming Category 2 plants (Aceraceae, Betulaceae, Fagaceae, Juglandaceae, Rosaceae, Salicaceae, and Solanaceae) show a significant positive correlation of midgut pH with diet guild rank (filled circles, $R^2 = 0.42$, $P < 0.05$, $y = 0.38x + 7.70$). Midgut pH values of all species consuming Category 1 plants (conifers, Cruciferae, Euphorbiaceae, Graminea, Malvaceae, and *Crotalaria*) are not correlated with diet guild (open circles, $R^2 = 0.006$, $P > 0.69$, $y = -0.05x + 8.25$, $N = 27$), whereas midgut pH values of species of Noctuoidea consuming Category 1 plants are negatively related to diet guild (filled squares, $R^2 = 0.36$, $P < 0.05$, $y = -0.26x + 8.99$, $N = 12$). N-values are expressed as (guild, N) and are as follows: Category 1, (1;1, 2;8, 3;13, 4;5), Category 2, (1;2, 2;0, 3;3, 4;6), Noctuoidea, (1;1, 2;3, 3;4, 4;4). Data are from Berenbaum (1980).

DISCUSSION

The phylogenetic distribution of an alkaline larval midgut strongly supports the presence of this trait in the ancestral dipteran, with subsequent loss in the lineage leading to the highly derived muscomorphan Diptera. Waterhouse (1949) observed that all larval Lepidoptera, including predators and those that feed on wax, have alkaline midguts. Midgut pH values reported for Trichoptera, the sister group of the Lepidoptera, although variable, are consistently alkaline (Shinoda, 1930; Martin et al., 1981). These data suggest that the common ancestor of the lepidopteran/trichopteran clade most likely also possessed an alkaline midgut. An alkaline midgut in the ancestral Diptera and in the common ancestor of the Lepidoptera/Trichoptera clade strongly supports a model in which this trait originated in a common ancestor of all Mecopterida. This hypothesis can be supported or refuted by analyses of the cellular mechanisms giving rise to high midgut pH in the Diptera and Lepidoptera.

While phylogenetic distribution suggests that the presence of an acidic or an alkaline midgut may be determined primarily by phylogeny, as proposed by Waterhouse (1949), the present analysis strongly supports the hypothesis that

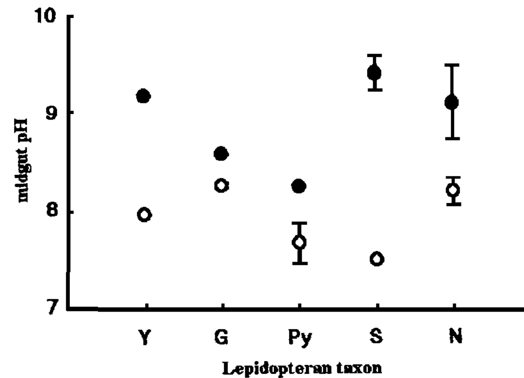

FIG. 5. Within each superfamily, species consuming conifers, Cruciferae, Euphorbiaceae, Graminea, Malvaceae, and *Crotalaria* (category 1, open circles) have lower reported midgut pH than do species consuming Aceraceae, Betulaceae, Fagaceae, Juglandaceae, Rosaceae, Salicaceae, or Solanaceae (category 2, filled circles). Only taxa containing species in both categories are illustrated. Numbers in parentheses are *N* values by diet characteristic, denoted as (category 1, category 2). Y = Yponomeutoidea (1,1), G = Gelechioidea (2,1), Py = Pyraloidea (5,1), S = Sphingoidea (1,2), N = Noctuoidea (11,2). Data are from Berenbaum (1980).

adaptation plays a role in fine-tuning midgut pH values to characteristics of the diet. Among lepidopteran superfamilies, phylogeny, diet guild, and midgut pH are inextricably linked. The success in predicting lepidopteran superfamily midgut pH by ranking diet guilds in the order fruit < grasses < herbs < trees and shrubs suggests that the magnitude of alkalinization is related to the relative amount and type of plant secondary compounds. In particular, diet guild rank is related to levels of tannins and polyphenols. Tannins are found at higher levels in trees than in herbs and are even lower in grasses (Bernays and Chapman, 1994). Polyphenols tend to be found in lower concentrations in fruits than in foliage (Felton et al., 1989), while most protein available to detritivores is tightly bound to polyphenols such as tannins and lignins (Suberkropp et al., 1976). The significant positive relationship of midgut pH and diet guild rank among insects feeding on plant materials containing polyphenols supports the hypothesis of Berenbaum (1980) that tannins drive the evolution of high pH. The possible adaptive benefits of high midgut pH in herbivores consuming tannins is still controversial, however (Feeny, 1970; Berenbaum, 1980; Felton and Duffey, 1991; Martin et al., 1987).

Despite the strong relationship between diet guild rank and midgut pH at the superfamily level, diet guild fails to predict midgut pH at the species level because different relationships exist between diet guild rank and midgut pH

within the two categories of plants revealed in the analysis. A positive relationship exists between midgut pH and diet guild rank among species in category 2 (Solanaceae, Fagaceae, Rosaceae, Aceraceae, Betulaceae, Juglandaceae, and Salicaceae), whereas a negative relationship exists among species (when the analysis is limited to the Noctuoidea) in category 1 (conifers, Euphorbiaceae, Graminea, Cruciferae, Malvaceae, and *Crotalaria*). This negative relationship appears to be driven primarily by elevated midgut pH in Noctuoidea feeding on grasses (guild rank 2) relative to other noctuoid species. The division of plant taxa into two categories on the basis of herbivore midgut pH values must be due to differences in allelochemicals between plants in the two categories. The plant taxa in category 1 are characterized by a variety of compounds. Conifers, Euphorbiaceae, and Malvaceae contain high levels of terpenes (Gershenzon and Croteau, 1991), Cruciferae are characterized by glucosinolates, which are broken down into isothiocyanates upon ingestion (Louda and Mole, 1991), and grasses contain DIMBOA (Felton, 1996). The plant taxa in category 2 are all described by Berenbaum (1980) as high in tannins, with the exception of members of the Solanaceae, which contain high levels of chlorogenic acid (Felton and Duffey, 1991). The distributions of these compounds are thus consistent with the two categories of plant taxa, whereas the distributions of coumarins (Berenbaum, 1991), cardenolides (Malcolm, 1991) and lectins (Leiner, 1991) are clearly not.

The terpenes, isothiocyanates, and DIMBOA appear to undergo similar chemical reactions. Many terpenes contain aldehyde groups, which are highly reactive at alkaline pH (Fieser and Fieser, 1961). Reactions of aldehyde groups at high pH include condensation reactions with other aldehydes to form complex resins and binding to the carbon next to carboxy-, nitro-, or nitrile groups (the Knoevenagel reaction; Fieser and Fieser, 1961) and with amines (Gershenzon and Croteau, 1991). Terpenes containing aldehyde groups are thus likely to reduce the bioavailability of proteins, and indeed this is believed to be the mode of action of gossypol (Gershenzon and Croteau, 1991). Similarly, isothiocyanates (formed from glucosinolates) bind to amino and sulfhydryl groups of proteins, decreasing bioutilization of nitrogen by rats, a reaction that is also enhanced at high pH (Hernandez-Triana et al., 1996). DIMBOA, found in grasses (category 1) undergoes chemical reactions similar to those of the terpenes and isothiocyanates (Felton, 1996). Interestingly, legumes contain tannins, yet midgut pH values of herbivores feeding on legumes were not significantly different from those feeding on either category 1 or category 2 plants. It was noted, however, that legumes of the genus *Crotalaria* contain pyrrolizidine alkaloids, which undergo chemical reactions similar to those of terpenes and isothiocyanates (Felton, 1996). *Utetheisa pulchella*, a member of the Noctuoidea that feeds on *Crotalaria*, has a reported mean midgut pH of 8.25, while two other lepidopteran species (*Papilio demoleus*; Papilionoidea, and *Datana integerrima*; Noctuoidea) that feed on legumes (presumably non-*Crotalaria*) have mean reported midgut

pH values of 9.0 and 9.5, respectively (Berenbaum, 1980). Similarly, conifers contain both tannins and terpenes, yet species feeding on conifers have low midgut pH. Of particular interest is *Lymantria monacha* (Noctuoidea), which feeds on pines (category 1) in addition to members of the Rosaceae and Fagaceae (category 2); it has a reported midgut pH of 8.3. Low midgut pH in species feeding on *Crotalaria* and on conifers and in *Lymantria monacha* suggest that selection for low pH in the presence of compounds characteristic of category 1 plants, such as terpenes and pyrrolizidine alkaloids, may override selection for high pH in response to tannins and drive selection for lower midgut pH. This interpretation is supported by the significant negative correlation between diet guild and midgut pH among Noctuoidea feeding on plants in category 1. It thus appears that low midgut pH (<9) may be an adaptive response to avoid the increased reactivity of terpenes, glucosinolates, isothiocyanates, and DIMBOA at high pH values. These hypotheses must be tested experimentally.

With the exception of the extreme generalists, species feeding on multiple taxa tend to limit their host-plant ranges to either members of category 1 or category 2, suggesting that host-plant range may be limited in part by midgut pH. A phylogenetic analysis of the plants in categories 1 and 2 was not performed, however, and this distribution of host-plant ranges may be the result of other factors. Extreme generalists may have arrived at novel strategies for overcoming the adverse effects of various allelochemicals under conditions of suboptimal matching of midgut pH to diet.

The Brachyceran families in this analysis are all members of the cyclorrhaphan Diptera. The ancestor of this group is believed to be saprophagous, ingesting large numbers of bacteria (Espinoza-Fuentes and Terra, 1987; Lemos and Terra, 1991a). Greenberg (1968) first demonstrated that the acidic region of the larval cyclorrhaphan midgut was responsible for the destruction of the majority of ingested bacteria. Acidic conditions within the midgut are hypothesized to have evolved in conjunction with the change to a saprophagous diet (Espinoza-Fuentes and Terra, 1987; Lemos and Terra, 1991a). It is not clear, however, how much more effective at destruction of bacteria a midgut region of pH 3–4 would be than a region of pH 11, such as that found in the midgut of *Tipula* (Martin et al., 1980). Perhaps the alkaline midgut was lost prior to the evolution of the saprophagous diet.

An exhaustive search of the literature for data on midgut pH values of nonmecopteridan insects was not performed, as this issue is beyond the scope of the present study; however, it is possible that the alkaline midgut of larval Mecopterida actually arose in larvae of the ancestral endopterygote. A cursory evaluation of the literature suggests that this hypothesis cannot be addressed with the available data. The most suitable outgroup for the analysis of midgut alkalinization within the mecopterida is the Hymenoptera (Hennig, 1981). Data obtained from the literature by the author on hymenopteran midgut pH values

are limited to a single report of an acidic midgut in the family Apidae (Schumaker et al., 1993). Apidae is a member of the derived hymenopteran suborder Apocrita, which has diverged from the ancestral hymenopteran condition to the extent that the larval gut is not complete but forms a blind-ended sac (Naumann, 1991). It therefore seems unlikely that Apidae would retain the ancestral hymenopteran midgut pH. Of greater interest would be the larvae of the more primitive phytophagous suborder Symphyta (Naumann, 1991). More distantly related to the Mecopterida are the Coleoptera. Reported midgut pH values were found for larvae belonging to two infraorders, the Scarabaeiformia and the Cucujiformia (Terra and Ferreira, 1994, Lawrence and Britton, 1991, Crowson, 1960). Larvae of the Scarabaeiformia have highly alkaline midguts (Bayon, 1980), while larvae of the more derived infraorder Cucujiformia, including the superfamilies Cerambycidoidea, Tenebrionoidea, and Chrysomeloidea (Lawrence and Britton, 1991, Crowson, 1967) have slightly acidic midguts (reviewed in Terra and Ferreira, 1994). Despite the availability of data for larvae of a number of species, the grouping of these species into only two infraorders prevents the inference of the ancestral state of coleopteran midgut pH. An in-depth phylogenetic analysis of larval midgut pH within the endopterygotes, especially the Hymenoptera, Coleoptera, and Neuropterida, coupled with mechanistic analyses of alkalinization in disparate taxa, is necessary to fully resolve the question of homology of midgut alkalinization among larval endopterygotes. The majority of exopterygotes have midguts that are mildly acidic (Terra and Ferreira, 1994), and incidences of midgut alkalinization among exopterygotes (Cooper and Vulcano, 1997) are therefore most likely the results of convergent evolution.

In conclusion, the analyses presented here have led to a number of hypotheses concerning the roles of phylogeny and diet in explaining the distribution of midgut pH among members of an insect clade, the Mecopterida. In particular, the data support the hypothesis that an alkaline midgut is a primitive characteristic of larval mecopteridan insects. Adaptive radiation within this group appears to have preserved or even enhanced this characteristic in species that feed on diets characterized by high levels of tannins or chlorogenic acid, whereas adaptation to diets containing terpenes, DIMBOA, glucosinolates, and pyrrolizidine alkaloids appears to have led to an evolutionary decrease in midgut pH. A variety of factors influence host plant range (Ehrlich and Murphy, 1988). The grouping of folivorous species by midgut pH into two distinct groups and the failure of species in the data set (with the exception of extreme generalists and *Lymantria monacha*) to utilize members of more than one hostplant category suggest that herbivorous species may be constrained to host-plant taxa for which they are preadapted in part by possession of a relatively high or low midgut pH. On the other hand, when both species feeding on category 1 and species feeding on category 2 are present in a lepidopteran superfamily, those species feeding on plants in category 2 always have higher pH than those feeding on plants in

Category 1, suggesting that midgut pH can evolve rapidly once a dietary switch has been made to host plants protected by different chemistry.

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THE ROLE OF PLANT RAPIDLY INDUCED RESPONSES IN ASYMMETRIC INTERSPECIFIC INTERACTIONS AMONG INSECT HERBIVORES[1]

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Abstract—The role of induced responses of tomato, *Lycopersicon esculentum*, in interspecific interactions between two polyphagous herbivores, the silverleaf whitefly, *Bemisia argentifolii* (WF), and the vegetable leafminer, *Liriomyza trifolii* (LM), was characterized in laboratory and field experiments. Feeding by LMs and WFs induced local and systemic production of putative defensive proteins, i.e., chitinases, peroxidases, β-1,3-glucanases, and lysozymes. The magnitude of the induction for each defensive protein varied between species. Unlike WFs, LMs caused a 33% local reduction in total foliar protein content. In a whole-plant choice experiment, adult LM feeding, oviposition, and larval survival were reduced by 47.7%, 30.7%, and 26.5%, respectively, for the WF-infested host compared with the controls. Early WF infestations also had negative systemic (plant-mediated) effects on LMs. Adult LMs preferred leaves from control plants to leaves of plants that had been previously infested with WFs; no reciprocal effect of LMs on WFs were found. Feeding by *Helicoverpa zea* larvae, which has been shown previously to affect LM performance, had no effect on WF survival and development. LM natural population dynamics were monitored on WF-preinfested and control plants in a field experiment. WF-infested plants were less suitable for LM development with an overall 41% reduction in LM population density. These results demonstrate asymmetric direct and plant-mediated interspecific interactions between generalist herbivores feeding simultaneously on the

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same host. Possible mechanisms by which WFs overcome plant defenses are suggested. This ability may also contribute to WF success that makes them a major pest worldwide. The study supports the idea that over an evolutionary time scale, herbivores sharing the same host plant will automatically compete.

Key Words—Herbivory, induced response, interspecific interactions, leafminers, pathogenesis related proteins, plant defense, tomato, whiteflies.

INTRODUCTION

The importance of interspecific competition affecting populations of insect herbivores has drawn much attention and debate among ecologists (e.g., Strong et al., 1984; Denno et al., 1995). Janzen (1973) suggested that, considering the evolutionary time scale, insect herbivores sharing the same host plant (resource) will automatically compete. Thus, in addition to direct interference and exploitative interactions among sympatric herbivores, interactions should include insects that do not interact directly due to spatial and/or temporal separation, i.e., indirect effects mediated by the host plant (Strauss, 1991; Wootton, 1994). There are few mechanisms by which different herbivorous insects may indirectly affect each other via the host plant. Changes in plant primary and secondary metabolites induced by an insect may alter nutritional quality and palatability, increase toxicity, and alter anatomy, phenology, and physiology of the host plant. These changes may affect other herbivores (Haukioja, 1990; Masters et al., 1993; Tallamy and Raupp, 1991; Inbar et al., 1995). Price et al. (1980) proposed that indirect competition among herbivores may act via the third trophic level, the natural enemies. Natural enemies may be attracted to chemical and physical signals generated in damaged plants following insect herbivory.

Induced responses of host plants refer to biochemical, physiological, and developmental changes in plants following stimuli originating from abiotic and biotic factors. It is now well established that some of the components of herbivore-induced responses are defensive and can negatively affect insect herbivore populations (Karban and Myers, 1989; Tallamy and Raupp, 1991; Karban and Baldwin, 1997; Fowler and Lawton, 1985). It is important to note that host-plant-induced responses are not necessarily negative (induced resistance) and could actually result in induced susceptibility for herbivory (Danell et al., 1985; Karban and Baldwin, 1997; Martinsen et al., 1998). Timing of plant responses and insect phenology are important in that they may determine herbivore fate. Preformed-induced responses are immediate but are locally restricted around the damaged areas. A rapidly induced response occurs within hours or days and may be localized around the damaged tissue or may be systemic in undamaged tissue. Delayed induced responses are changes in plants in the following season or year(s) (Baldwin, 1994). Here we shall refer to the induced response in infested

leaves as a local induced response; induced responses in noninfested leaves on infested plants will be referred to as a systemic response.

Many plant responses, especially to insect herbivory, are rather general (i.e., not species specific) and, therefore, may have a negative effect on the inducers themselves as well as on other herbivores (Baldwin, 1994; Karban and Myers, 1989; Stout and Duffey, 1996; Agrawal, 1998). We have examined how host-plant-induced responses to one herbivore change the host-plant suitability for another cofeeding insect herbivore while the inducer itself remains apparently unaffected by those changes.

The sympatric feeding of the silverleaf whitefly (WF), *Bemisia argentifolii* Bellows and Perring (Homoptera: Aleyrodidae), and the leafminer (LM), *Liriomyza trifolii* (Burgess) (Diptera: Agromyzidae), on tomatoes, *Lycopersicon esculentum*, provides us with an excellent opportunity to address the role of induced host-plant responses in interspecific interactions among generalist herbivores. Both WFs and LMs are polyphagous and occasionally feed simultaneously on tomatoes. Feeding by LMs and WFs induces a variety of defensive phytochemicals including pathogenesis-related (PR) proteins (Stout et al., 1994; Mayer et al., 1996). PR proteins such as peroxidase, glucanase, and chitinase are induced in pathological or related situations including herbivory and are thought to have defensive roles in the plant (van Loon et al., 1994). Induced responses in tomatoes may effectively reduce insect herbivore performance (Stout et al., 1994; Stout and Duffey, 1996; Inbar et al., 1998). Several biological characteristics of the LM and WF limit the possibilities of direct interference between them and, consequently, emphasize the role of the host plant as a mediator of interspecific interactions. Adult and immature WF stages are phloem feeders that feed predominantly on the abaxial side of host-plant leaves. In contrast, adult LMs feed and oviposit almost entirely on the adaxial surface of the leaves. LM larvae mine the adaxial side of the leaves, feeding on the palisade and spongy mesophyll (Parrella, 1987). Larval mobility of both species is limited, reducing the chance of direct interference. WF nymphs are stationary, with limited movement during the first instar, the crawler. LM larval mobility is restricted to the mines. As far as we know, these species do not share common natural enemies.

Here we have addressed the following questions:

1. Do WFs and LMs affect each other when feeding on the same host plant?
2. If WFs and LMs do affect each other, is the effect mediated by the plant (localized and/or systemic)?
3. If interspecific interactions are found, do they correlate with the induction of plant biochemical responses (e.g., PR proteins) and foliar protein levels?
4. Do these interspecific interactions alter herbivore population dynamics in the field?

METHODS AND MATERIALS

Host Plants and Insect Culture. Tomato (*Lycopersicon esculentum* cv. Agriset) plants were 4–5 weeks old and grown in 5.7-cm pots with Metro Mix 500 growing medium (Grace, Sierra, California). All plants were initially treated with a fungicide (0.4 g/liter; Bayleton, Bayer Corp., Kansas City, Missouri) and fertilized weekly. WFs were from a colony of *B. argentifolii* that was maintained in a greenhouse on collards and tomatoes. Lack of external symptoms on the plants indicated that the colony was apparently free of pathogenic viruses. The LMs used in this study were from a colony initiated in 1983 and maintained on cowpea, *Vigna sinensis* (Stickm.).

Preinfestation Protocol. When experiments required early insect infestation, plants were placed in the LM or WF colonies for three days. Subsequently, all adult insects were removed from the plants by shaking and aspiration; no attempt was made to remove the immature stages. LM preinfestation resulted in 0.43 ± 0.08 mines/cm^2. WF preinfestation resulted in 6.1 ± 1.9 nymphs/cm^2. After removal of adult insects, preinfested plants were transferred to a different greenhouse together with the control (insect-free) plants. Immature stages were allowed to develop on the preinfested plants for 14 days (LM, reached the pupal stage), and 20 days (WF, reached the fourth instar). This was sufficient time for these insects to induce host-plant biochemical responses (Stout et al., 1994; Mayer et al., 1996), and it eliminated the problem of having adults (from the preinfested generation) in the experimental systems. By the end of the preinfestation period, each plant had six fully expanded, infested leaves (with mines or WF nymphs) and an additional one to three unexpanded leaves. Control plants had similar numbers of leaves that were insect-free.

Effect of WFs on LM Preference and Performance. Twenty-four randomly assigned pairs of control and WF-preinfested plants were each placed in buckets (15.2-liter capacity; one pair/bucket) sealed with cellophane. Five male and five female newly emerged adult LMs were introduced into each bucket with an aspirator. Adult LMs were allowed to feed and oviposit for 24 hr and then removed with an aspirator. Each plant was placed separately in a plastic dish to allow convenient collection of the developing LM larvae that fell off the leaves 12 days later (to pupate). Adult LM host preference was measured by counting the number of oviposition and feeding punctures per leaf and per square centimeter. The oldest leaf was designated leaf 1, the second from the bottom as leaf 2, etc. Collected puparia were considered as individuals that successfully completed their development. Thus, larval survival rates were calculated by dividing the number of puparia by the number of egg punctures. The data were analyzed by the paired comparison t test on square root transformed data.

Effect of LM Preinfestation of WFs Oviposition Preference. Control and LM-preinfested plants ($N = 20$ each) were placed in the greenhouse with the WF

colony on a 1-m-high bench in a complete randomized design. After three days, WF eggs were counted on two leaf disks (2 cm^2) sampled from the terminal trifoliate of all leaves (1–9) by using a stereomicroscope. Two-way ANOVA was used to test the effect of LM preinfestations on WF host preference, with preinfestation and leaf position as main effects.

Systemic Effect of Early WF Infestation on Sequential LM Preference and Performance. Gauze sleeves were sealed around the top two leaves (7 and 8) and the apical bud. Half the plants were preinfested with WFs. After removal of the adult preinfesting WFs, the sleeves were removed, and the control and experimental plants were placed together for an additional 20 days. Subsequently, all six bottom leaves were removed with a razor blade to prevent any direct interspecific interactions. The control plants were treated similarly; all plants had the three top WF-free, fully expanded leaves (7, 8, and unfolded 9). Leaf excising might weaken herbivore systemic effects, but it was deemed necessary to eliminate any direct interactions including pheromonal agents. Recent studies demonstrated that artificial damage (such as leaf clipping) causes less induction compared with that produced by herbivory, probably because of the reduced actual leaf area damaged and the effects of insect saliva (Alborn et al., 1997; Agrawal, 1998, and references therein). Furthermore, excising tomato leaflets did not overwhelm the induced responses to herbivores (Stout and Duffey, 1996). Control and WF-infested plants were randomly divided into 24 pairs and challenged with adult LMs in buckets as described earlier (see WF effect on LM at whole plant level).

Effects of Induced Systemic Response by Corn Earworm on WF Performance. Stout and Duffey (1996) demonstrated that induced responses of tomato foliage following feeding damage by larvae of corn earworm, *Helicoverpa zea* Boddie, have a negative effect on subsequent herbivores (LMs and the beet armyworm, *Spodoptera exigua*). The most pronounced effects were documented when systemic induction was tested at the leaf level, i.e., different leaflets within the same leaf (Stout et al., 1994; Stout and Duffey, 1996). Since we did not find any effect of LMs on WFs in the whole plant experiment (see Results), we repeated part of Stout and Duffey's (1996) experiment to determine whether or not WF fitness would be affected by a strong induced response that results from *H. zea* feeding. A single second instar of *H. zea* (obtained from the USDA, ARS, CMAVE, Gainesville, Florida) was restricted to the terminal leaflet of leaf 3 of 4-week-old plants with five leaves ($N = 20$) in clip cages (2.9 cm diameter). Larvae were allowed to feed for 24 hr and then removed. The control plants ($N = 20$) had empty clip cages placed identically for 24 hr. After an additional 48 hr, the terminal leaflet was excised with a razor blade. Ten adult WFs were allowed to oviposit for 1 hr (and then removed) to similar clip cages on the ventral side of a leaflet located two leaflets down from the terminal leaflet. WF performance was determined after four weeks. Opened nymphal cases (used as an indicator of adult WF emergence) and red-eyed nymphs (fourth instar) were counted.

Phytochemical Analyses. Another batch of controls and LM- and WF-preinfested plants that were treated similarly ($N = 15$ each) were used to detect local and systemic induction of PR proteins. The top two leaves (7 and 8) were covered with gauze sleeves to prevent LM feeding and oviposition; control plants were treated likewise. Leaves 4 and 7 of each plant were used for biochemical analyses as represented for local and systemic responses.

Fresh samples were weighed prior to protein extraction. Leaf samples were crushed with an electric roller grinder (Ravenel Specialties Co., Seneca, South Carolina). The extracts were washed from the rollers with 20 ml of 0.1 M sodium phosphate (pH 7.4) into tubes containing 0.6 g of hydrated polyvinylpolypyrrolidone (Sigma Chemical Co., St. Louis, Missouri). The tubes were capped and mixed for 30 min at 4°C. The samples were centrifuged at 20,000*g* for 15 min. The supernatant was filtered through a layer of Miracloth (Calbiochem, La Jolla, California) into dialysis tubing (Spectrum, Laguna Hills, California) with a molecular weight cutoff of 6000–8000 Da. The samples were dialyzed overnight in distilled water and subsequently lyophilized. The dried samples were resuspended in 3 ml of water and centrifuged for 10 min at 10,000*g*. The resulting supernatant was used for analyses. Total protein, chitinase, peroxidase, β-1,3-glucanase, and lysozyme levels were measured as described previously (Mayer et al., 1996). These proteins were selected for their potential defensive role against herbivores.

We calculated ANOVA in a split plot design with preinfestation as the main plot and leaf position on the plant as the subplot to test the local and systemic induction of PR proteins by LMs and WFs. Although this design sacrifices precision in estimating the main plot effects, it improves comparison of the subplot (systemic) effects and provides a tool to examine the interaction between the main effects. Since systemic and local responses are different (see Results and Stout et al., 1996), a mean separation (LSD) test was conducted for each protein and each leaf at positions 4 and 7 (Little and Hills, 1978). Each enzyme for LMs and WFs was tested separately in the SAS CD statistical package (SAS Institute, 1988). All data on enzyme activities were transformed by $\log_{10}(x + 1)$ before analysis.

Field Experiment. Control and WF-preinfested plants were transplanted to field plots at the University of Florida, Indian River Research and Education Center, Fort Pierce, Florida. Raised beds 15 cm high × 1.06 m wide were spaced at 2.13-m bed centers. Plants were set 0.6 m apart in the center of each bed. Beds were covered with black polyethylene mulch and watered with subsurface irrigation. Plots, each with 10 plants, were replicated seven times in a completely randomized design. The plants were not treated with any pesticides at any time. Preinfested plants contained WF nymphs and eggs when transplanted (see preinfestation protocol). WF and LM natural infestations and movements between plants were not interfered with after transplanting.

The terminal trifoliate of the seventh leaf from the top (a different leaf was sampled every week) of all plants was sampled weekly to determine insect population densities. The number of mines on the adaxial surface of the terminal leaflets represented LM density (Schuster and Beck, 1992). The number of WF nymphs on the entire abaxial surface of the same leaflet was used to monitor WF density. Leaflet area was measured after counting with a leaf area meter (LI 3000, Lambda Instruments Corp., Lincoln, Nebraska). Data were analyzed by a repeated measurement ANOVA with date and preinfestation as the main effects. All sets of data of LM and WF eggs, nymphs, and adults were square-root-transformed before analysis. This trial examines the overall effect in the field and does not distinguish between direct and plant-mediated mechanisms.

RESULTS

Effect of Early WF Infestations on LM Preference and Performance. Adult LM females preferred the control plants to WF-preinfested plants (Table 1). WF preinfestations resulted in a 30.6% reduction in the number of eggs laid per plant, which was almost twofold lower on an area comparison. WF preinfestations also reduced the number of LM feeding events and the number of leaves and leaflets used by LMs for feeding and oviposition compared with the control plants (Table 1). The survival of LM larvae on the control plants was 26.5% higher than on WF-preinfested plants (Table 1). Almost no WF nymphs were observed on the youngest unfolded leaves (7–9) of the preinfested plants. Regardless of the presence of WFs, LM female activities (Figure 1) were concentrated in the middle-position leaves of the plants. Peak feeding activity of adult LMs on the control plants was on leaf 4, while on the WF-preinfested plants these activities peaked

TABLE 1. EFFECT OF WF PREINFESTATIONS ON ADULT LM PREFERENCES AND LARVAL PERFORMANCE[a]

Variable	Control plants	WF-infested plants	Paired t
Eggs/plant	18.6 ± 2.7	12.9 ± 4.4	2.32*
Eggs/cm^2	0.52 ± 0.07	0.36 ± 0.09	2.01*
Feedings/plant	58.8 ± 14.4	30.7 ± 14.1	2.34*
Leaves with eggs	2.9 ± 0.3	2.3 ± 0.4	1.7 NS
Leaflets with eggs	7.0 ± 0.9	4.4 ± 1.2	2.6*
Leaves used for feeding (N)	3.04 ± 0.32	2.0 ± 0.25	3.2**
Leaflets used for feeding (N)	8.1 ± 1.2	3.9 ± 0.6	3.8**
Larval survival (%)	33.9 ± 4	34.9 ± 4.1	2.41*

[a]Values are the means ± SE, $N = 24$ (pairs). NS = not significant; $^*P < 0.05$, $^{**}P < 0.01$.

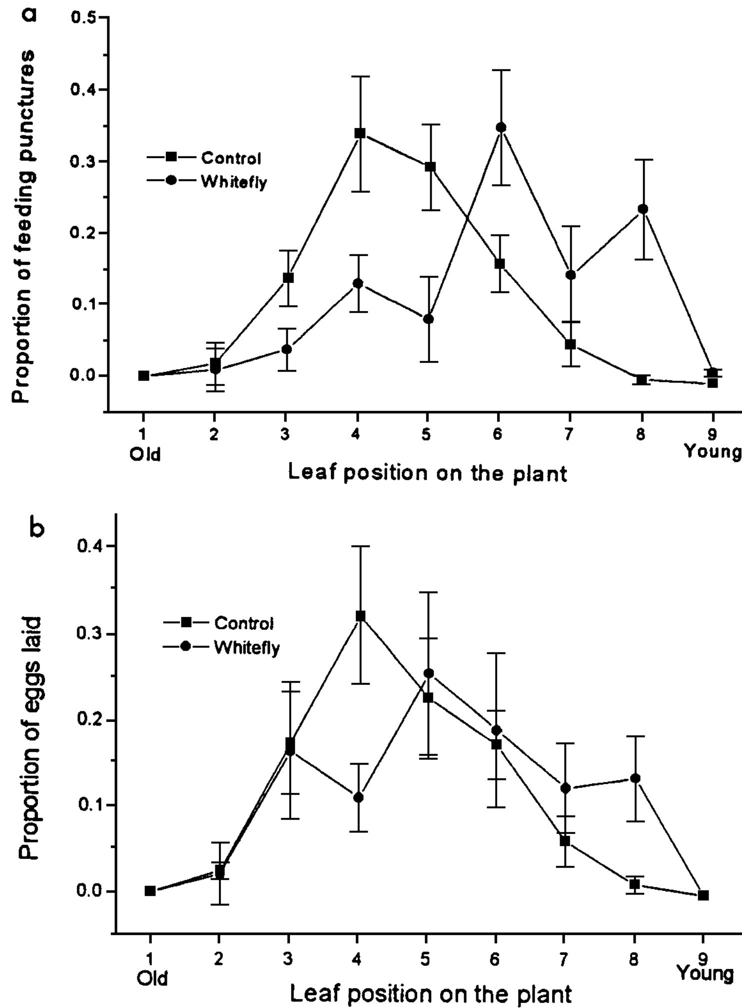

FIG. 1. Distribution of LMs among leaves of WF-preinfested and control plants in the whole plant experiment: (a) proportion of leafminer feeding, and (b) oviposition punctures. Total numbers of LM preference are given in Table 1. Values are means ± SE.

more distally on leaf 6 (Figure 1a). The distribution of oviposition was similar on both host plants (Figure 1b).

Effect of LM Preinfestation on WFs Oviposition Preference. Since LM activity is concentrated in the middle, fully expanded leaves of the plant, most of the preinfested mines were located on leaves 3–5. Two-way ANOVA indi-

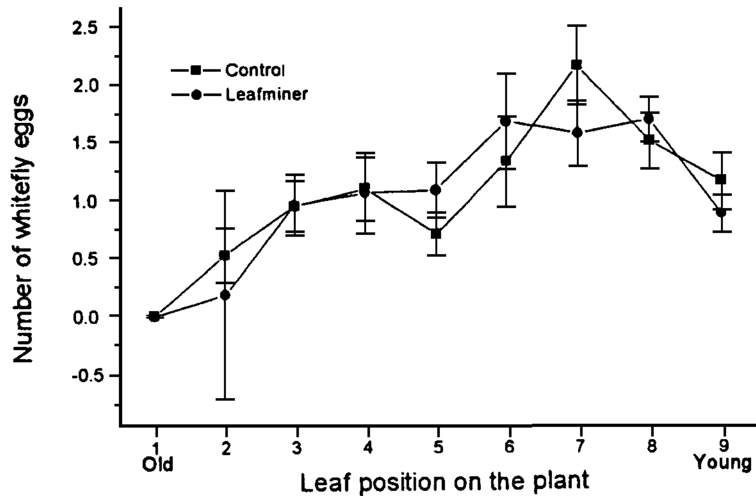

FIG. 2. Effect of LM preinfestation on WF oviposition preference. Young leaves (7–9) of the LM-preinfested plants were mine-free. Values are mean numbers of eggs per square centimeter ± Se.

cated that LM preinfestations did not affect WF adult oviposition ($F_{1,342}$ = 0.45, P = 0.53, Figure 2). WFs preferred to oviposit on younger (6–8) rather than on older leaves, regardless of LM preinfestation ($F_{8,342}$ = 14.34, $P \ll 0.01$, Figure 2). Consequently, there were no LM × leaf position interactive effects on WF preference ($F_{8,342}$ = 1.24, P = 0.27). Within the LM-preinfested plants, we found no correlation between the number of mines and the number of WF eggs (r = 0.12, P = 0.66; data not shown). These results indicate that WF host selection was not influenced by LM preinfestation.

Systemic Effect of Early WF Infestation on Sequential LM Preference and Performance. Significant reductions in LM oviposition (24%) and feeding (27%) incidents were found on leaf 7 on the WF-preinfested plants (Table 2). Although we found a 25% reduction of LM survival on leaf 7 of the WF-preinfested plants, the data were not significant. The systemic effect on WFs on LMs was found to be limited to the proximal noninfested leaf only. No significant effects were detected on leaf 8 located two leaves above the WF-preinfested leaves.

Effects of Induced Systemic Response by Corn Earworm Caterpillars on WF Performance. The mean number of WF eggs laid on the control (13.1 ± 1.9) and *H. zea*-fed leaves (12.3 ± 2.95) was similar (Mann-Whitney U test, Z = 0.907, P = 0.36). On both the experimental and control plants, more than 98% of the eggs hatched. WF nymph development was not affected by systemic responses caused by prior feeding of *H. zea* (Figure 3).

TABLE 2. SYSTEMIC EFFECTS OF WF PREINFESTATION ON LM PREFERENCE AND PERFORMANCE[a]

	Eggs/cm^2	Feed/cm^2	Larval survival
Leaf 7			
Control	0.37 ± 0.04	0.77 ± 0.06	0.29 ± 0.03
WF	0.28 ± 0.04	0.56 ± 0.04	0.16 ± 0.04
Paired t	2.23*	2.32*	0.98 NS
Leaf 8			
Control	0.31 ± 0.04	0.56 ± 0.03	0.13 ± 0.04
WF	0.26 ± 0.04	0.51 ± 0.05	0.17 ± 0.07
Paired t	1.08 NS	0.89 NS	0.33 NS

[a]Since the last leaf along the shoot axis that was preinfested was leaf 6, any effects detected on leaves 7 and 8 were systemic. Values are the means ± SE and the results of the paired t test (N = 24 pairs). NS = not significant, $^*P < 0.05$.

Phytochemical Analyses. Overall, infestation of both species induced higher levels of PR proteins. Irrespective of the species of herbivore, induced and constitutive levels of PR proteins were higher in old leaves (leaf 4) compared with young ones (Tables 3 and 4).

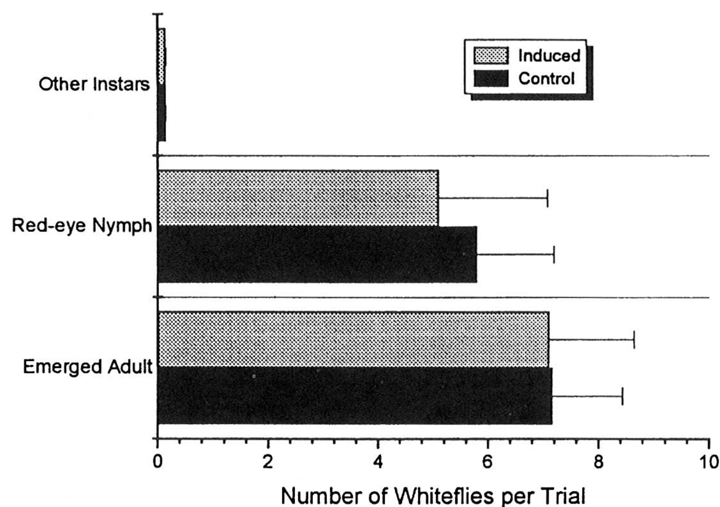

FIG. 3. Effect of induced plant systemic response to *H. zea* larval feeding on WF survival and development. Red-eye nymphs (pupa) are the last stage (fourth instar) in WF development. Empty nymphal cases indicate that an individual successfully completed its development and emerged as an adult. Other instars are individuals that remain as eggs or as instars 1–3. Values are means ± SE.

TABLE 3. EFFECTS OF WF PREINFESTATION ON LOCAL AND SYSTEMIC INDUCTION OF PR PROTEINS IN TOMATOES[a]

Treatment	Total protein (mg/g tissue)	ΔA_{510}/min/g tissue			β-1,3-glucanase (mmol/min/g tissue)
		Peroxidase	Lysozyme	Chitinase	
Local (leaf 4)					
Control	0.90 ± 0.06a	21.27 ± 1.49a	328.83 ± 34.41a	3.89 ± 0.19a	0.95 ± 0.17a
WFs	1.11 ± 0.14a	22.92 ± 1.98a	425.36 ± 46.78b	4.31 ± 0.16b	2.22 ± 0.16b
Systemic (leaf 7)					
Control	1.54 ± 0.13a	9.87 ± 1.96a	81.80 ± 26.62a	1.94 ± 0.25a	0.21 ± 0.03a
WFs	1.27 ± 0.11b	19.92 ± 1.39b	295.92 ± 24.12b	4.19 ± 0.22b	0.81 ± 0.11a
			F value		
Split plot ANOVA, source of variation					
Treatment (WF)	0.18 NS	14.81***	32.94***	31.21***	57.51***
Leaf position	28.41***	25.21***	45.42***	39.46***	91.44***
Treatment × position	66.75***	26.30***	18.58***	43.29***	0.71 NS

[a]Leaf 4 (in the treated plants) was preinfested with WFs and thus represents local induction. Leaf 7 was not exposed to WFs at any time and thus reflects systemic induction. Values are the means ± SE ($N = 15$). The results of the split-plot ANOVA test (F values) for each enzyme are given at the bottom. Similar letters within pairs indicate nonsignificant mean separation (LSD). NS = not significant, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

TABLE 4. EFFECTS OF LM PREINFESTATION ON LOCAL AND SYSTEMIC INDUCTION OF PR PROTEINS IN TOMATOES[a]

Treatment	Protein (mg/g tissue)	ΔA_{510}/min/g tissue			β-1,3-glucanase (mmol/min/g tissue)
		Peroxidase	Lysozyme	Chitinase	
Local (leaf 4)					
Control	3.01 ± 0.31a	33.93 ± 9.59a	107.23 ± 108.5a	3.69 ± 1.33a	0.21 ± 0.21a
WFs	1.98 ± 0.68b	67.25 ± 2.77b	422.36 ± 633.4b	4.62 ± 1.83a	0.41 ± 0.48a
Systemic (leaf 7)					
Control	0.45 ± 0.12a	9.78 ± 3.16a	87.8 ± 43.38a	1.49 ± 0.39a	0.56 ± 0.16a
WFs	0.58 ± 0.27a	8.51 ± 8.36a	65.6 ± 42.83a	2.70 ± 1.03b	1.06 ± 0.47b
			F value		
Split plot ANOVA, source of variation					
Treatment (LM)	12.01***	2.31 NS	4.75*	6.56*	10.77**
Leaf position	174.15***	68.27***	6.85*	26.08***	37.93***
Treatment × position	13.34***	11.51***	8.8**	NS	0.51 NS

[a]Leaf 4 (in the treated plants) was preinfested with LMs and thus represents local induction. Leaf 7 was not exposed to LMs at any time and thus reflects systemic induction. Values are the means ± SE ($N = 15$). The results of the split-plot ANOVA test (F values) for each enzyme are given at the bottom. Similar letters within pairs indicate nonsignificant mean separation (LSD). NS = not significant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Local and Systemic Response of Tomatoes to WFs. WFs feeding on tomato leaves induced higher levels of PR proteins locally and systemically on noninfested young leaves (Table 3). The levels of β-1,3-glucanase, chitinase, peroxidase, and lysozyme were increased 1.5- to 3-fold in the infested leaves (leaf 4) compared with the control leaves. Local induction of peroxidase was not statistically significant. All PR proteins that were measured after WF feeding increased systemically in young noninfested leaves (i.e., leaf 7); however, the systemic induction β-1,3-glucanase was not significant. Total protein levels were not significantly changed either locally or systemically (Table 3).

Local and Systemic Response of Tomatoes to LMs. As was found with WFs, LM feeding locally induced higher levels of the PR proteins, although only peroxidase and lysozyme were significant (Table 4). Only β-1,3-glucanase and chitinase were induced systemically in the young uninfested leaves (increased by approximately 50%). LM larvae that consumed the leaf mesophyll locally reduced foliar protein content by 37%, but the systemic effect was not significant.

Field Experiments. Differences in WF densities between control and WF-preinfested plants persisted for three weeks after transplanting and declined rapidly in week 4 ($F_{1,96}$ = 131.1, $P < 0.01$; Figure 4a). At four weeks, leaves 7 from the top no longer reflected the preinfestation period since they had expanded entirely after that period. From week 4 to the end of the experiment, WF populations on both control and experimental plants were low, with fewer than 0.1 nymphs/cm^2. Natural field infestations of LMs occurred quickly. Small mines were found one week after transplanting. LM density on control plants peaked two weeks after transplanting. Significantly lower LM densities were observed for weeks 2–6 after transplanting in the WF-preinfested plants compared to controls ($F_{1,96}$ = 23.62, $P < 0.01$). No significant differences were found in LM densities between the control and WF-preinfested plants after week 6 (Figure 4b). From week 4, the new leaves currently sampled (leaf 7 from top) had similar (and negligible) WF densities, while preinfested plants still had large WF populations on their basal old leaves.

DISCUSSION

Strong asymmetric relationships (amensalistic) were detected between WFs and LMs when they were feeding simultaneously on the same host plant. The effects of WFs on LMs were both direct (interference or exploitation interactions) and systemic. However, the systemic effect was restricted only to leaves located proximally to WF infestations. WF and LM feeding induces higher levels of PR proteins in tomato, although they differ in the magnitude of the local and systemic response for each enzyme. High WF infestations in the field resulted in the reduction of LM populations during young plant stages (two to six weeks after transplanting).

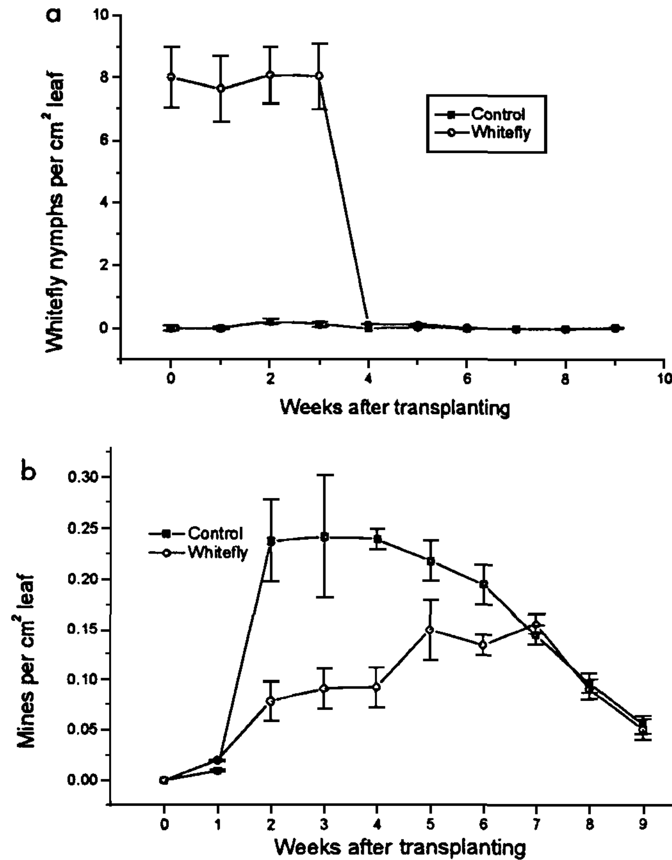

FIG. 4. (a) Field populations of WF nymphs on WF-preinfested and control plants. Values are means ± SE. (b) Effect of WF preinfestation on LM population dynamics in the field. Values are means ± SE.

Since induced plant responses to herbivory are thought to be general (Baldwin, 1994; Karban et al., 1987), most studies that examined systemic (rapid) induced responses reported negative effects on the inducing individual(s) or other conspecific and allospecific herbivores that feed simultaneously or shortly after (Hougen-Eitzman and Karban, 1995; Karban and Myers, 1989; Zangerl, 1990; Kogan and Fischer, 1991; Jones et al., 1993; Stout and Duffey, 1996). Therefore, the ability to use local and systemic induced responses to reduce competition between generalist insects feeding simultaneously on the same plant is probably constrained by the general negative effect of the host-plant responses. These effects can be avoided when the induced response is delayed, relative to the life-

span of the insect causing the induction. In this case, qualitative and quantitative changes take place after the insect has completed its development. Indeed, some of the most compelling evidence of induced-response-mediated asymmetrical interactions comes from species that are temporally separated (Faeth, 1986; Harrison and Karban, 1986; Haukioja, 1990). WFs appear to be able to overcome rapidly induced responses in tomatoes. This ability was observed regardless of source of induction, LMs or *H. zea* and it is in sharp contrast to other studies that reported that insect herbivores are severely affected by tomato-induced responses (Edwards et al., 1986; Duffey and Felton, 1991; Stout and Duffey, 1996).

Both LMs and WFs can induce local and systemic responses that differ in strength. Similarly, Stout et al. (1994) found that the ability of LMs to induce tomato responses was quantitatively mild compared with russet mites or *H. zea*. Differences in insect-specific abilities to induce host-plant responses have been found in several systems (Hartley and Lawton, 1991; Felton et al., 1994; Stout et al., 1994). We suggest that in our system the asymmetric relationship between WFs and LMs may stem from their differential ability to overcome plant defenses rather than their differential ability to induce specific responses. This conclusion is based on several pieces of evidence. First, the induced response triggered by *H. zea* feeding in tomatoes that was found to be harmful to LMs and leaf-chewing caterpillars (Stout and Duffey, 1996) did not affect WF survival and development. Second, young tomato leaves are also highly protected by constitutive chemicals, resulting in diminished herbivory on terminal leaves (Wilkens et al., 1996). In contrast, WFs are unaffected by these constitutive defenses and prefer young unfolded tomato leaves (Liu and Stansly, 1995). Finally, exogenous application of elicitors to tomatoes induced responses that lowered LM populations, but failed to affect WFs (Inbar et al., 1998). Nevertheless, differences in tomato-induced responses did vary between species, and we did not measure other possible defensive compounds (e.g., proteinase inhibitors and polyphenol oxidase). Thus, the role of specific tomato responses to each insect may also determine the outcome of the observed interaction.

How do WFs overcome tomato-induced responses? One explanation could be provided by the physiological traits used to detoxify plant defensive compounds. Additional explanations may be related to the insect feeding habits. WFs are phloem feeders (Cohen et al., 1996), while LM larvae feed in the palisade mesophyll (Parrella, 1987). Other insects that were found to be affected by induced responses in tomatoes are mainly leaf-chewing lepidopterans. It has long been suggested that phloem feeders are less exposed to plant chemical defense systems. Many plant toxins and other defensive compounds, including proteinase inhibitors in tomatoes (Walker-Simmons and Ryan, 1977), are thought to be stored in intracytoplasmic vacuoles in parenchyma and epidermal cells (McKey, 1979; Mullin, 1986; Berenbaum, 1991; Rosenheim et al., 1996). Vascular tissues should not be considered defenseless, but it is possible that fewer defensive com-

pounds and possibly their lower concentrations in the vascular system are less effective against phloem (and xylem) feeders (Rosenheim et al., 1996). Recently, Denno et al. (1995) recognized the importance of feeding guilds in insect herbivore competition. They found that leafminers are less successful when feeding on previously induced plants. However, they did not find clear evidence that sap feeders in general prevail in interguild interactions.

Tomato resistance is based on a complex of defensive systems that includes PR proteins, proteinase inhibitors, polyphenol oxidases, and phytoalexins (Duffey and Stout, 1996). The levels of several PR proteins that probably affect insect fitness were induced by WFs both locally and systemically. Chitinase degrades chitin and can consequently damage chitin-based structures such as the peritrophic membrane, which provides a physical barrier to ingested pathogens and other substances that pose a hazard to the insect. Chitinase activity may interfere with insect development, feeding, and growth; facilitate microbial infection; and finally cause death (e.g., Wang et al., 1996). Peroxidases are involved in production and polymerization of phenolics, lignification, and hypersensitive responses, thus affecting food digestibility and protein availability to herbivores (Bowles, 1990; Duffey and Stout, 1996). Peroxidase and chitinase genes have been introduced into several crops in an attempt to create transgenic insect-resistant plants (Carozzi and Koziel, 1997). Lysozymes and β-1,3-glucanases are defensive enzymes that protect plants against bacterial pathogens. Ingested lysozymes may affect insect symbiotic flora and interfere with insect digestion. Because WFs are phloem feeders, they may create a nutritional sink that diverts nutrients from neighboring leaves (Inbar et al., 1995) resulting in a reduction in LM performance. WFs also systemically reduce leaf photosynthetic efficiency (Inbar, Doostdar, and Mayer, unpublished data). However, total foliar proteins, which are important to LM development (Minkenberg and Ottenheim, 1990) were not significantly altered by WFs and, therefore, were not considered to be a major factor in the interactions observed.

Local effects of WFs on LMs were stronger than the systemic effects, which were temporally and spatially limited. The possible systemic effect of WFs lasted approximately two weeks and then disappeared in the field experiment, although the direct effects in the field have not been controlled. Similarly, in the greenhouse, WF systemic effects on LMs were pronounced near WF-infested leaves. Natural WF infestations cover most of a plant's leaves. Eggs are laid on the young upper leaves, and immature stages (nymphs and pupa) are distributed on middle and lower leaves (Liu and Stansly, 1995; Schuster, 1998). LMs, on the other hand, prefer mature middle leaves and do not shift to the young WF-free leaves (Minkenberg and Ottenheim, 1990). This is probably because of constitutive plant defense systems in young tomato leaves (e.g., soluble phenolics) (Wilkens et al., 1996) that had stronger effects on LM preference than the tomato-induced responses to WFs. Thus, the effects of WF natural infesta-

tions on LMs will be more detrimental and will involve direct and indirect (local and systemic) mechanisms, as were found in the whole plant experiment where LMs had no refuge leaves.

We believe that the effects reported in this study are more general, as a consequence of the keystone herbivore status (sensu Hunter, 1992) of WFs. Direct and plant-mediated mechanisms are involved in the negative effects of WF on cabbage loopers (*Trichoplusia ni*) when feeding on collards (Inbar et al., 1999). WFs are polyphagous pests that are found in many parts of the world, occasionally in high densities (Gerling and Mayer, 1996; Byrne and Bellows, 1991). There would be numerous occasions where they would share the same host plant and interact with other herbivores. The fate of the interactions between WFs and other herbivores will probably depend on the host plant, the mode of feeding of the potential competitor, WF density, and the timing of interactions.

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FIREFLY TOXICOSIS IN LIZARDS[1]

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Abstract—Ingestion of fireflies of the genus *Photinus* (Lampyridae) can be lethal to Australian lizards of the genus *Pogona* (Agamidae), probably because of the poisonous steroidal pyrones (lucibufagins) that these fireflies contain. One *Photinus* may suffice to kill a *Pogona*. Captive *Pogona* kept as pets need to be shielded from firefly ingestion. African chameleons (*Chamaeleo*; Chamaeleonidae) appear also to be vulnerable to *Photinus* toxicosis.

Key Words —*Pogona, Chamaeleo, Lacerta, Litoria (Pelodryas) Photinus,* lucibufagins, cardiotonic agents, pet industry.

INTRODUCTION

Fireflies of the genus *Photinus* are poisonous. Their bodies contain lucibufagins (Eisner et al., 1978), steroidal pyrones related structurally to such well-known toxins as the bufodienolides of toads and the cardenolides of plants (Fieser and Fieser, 1949; Budavari et al., 1996) (Figure 1). Not surprisingly, the lucibufagins protect *Photinus* against predation. Spiders (*Phidippus* spp.) are orally deterred by lucibufagins, as are birds (*Hylocichla* spp.), which also show reluctance to attack *Photinus* (Eisner et al., 1978, 1997). In the exceptional case where a

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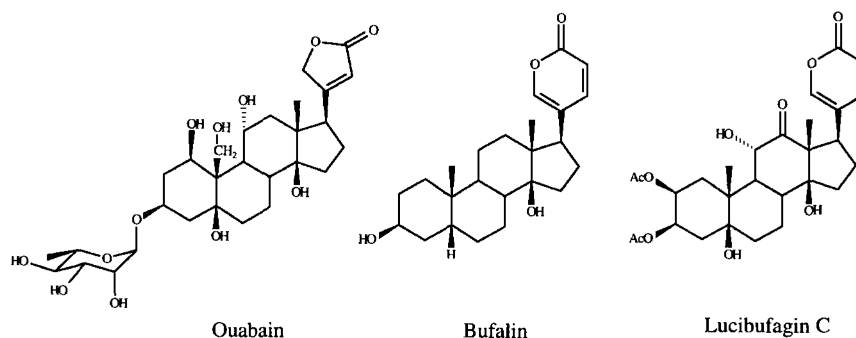

FIG. 1. Chemical structures of a cardenolide (ouabain), a bufodienolide (bufalin), and a lucibufagin.

Hylocichla was noted to ingest a *Photinus*, the bird reacted in short order by regurgitating it (Eisner et al., 1978). We know of no data on LD_{50} determinations for lucibufagins, but have now learned that ingestion of *Photinus* can be fatal to Australian lizards of the genus *Pogona*, commonly known as bearded dragons.

Pogona lizards are highly tractable and easily maintained in captivity; they are bred in large numbers in the United States for the pet market (Vosjoli and Mailloux, 1996). The two instances in which ingestion of *Photinus* led to death of *Pogona* involved lizards that were maintained as pets. Their case histories follow.

METHODS AND MATERIALS

Case 1. The owner of a healthy 8-month-old male *P. vitticeps* (about 100 g body mass) captured a number of fireflies one July evening in the environs of his home (Iowa City, Iowa) and offered these to the lizard in its cage (aquarium tank). The lizard promptly ingested several of the fireflies. Within about 30 min the lizard began exhibiting violent head-shaking movements, followed by pronounced and increasingly frequent oral gaping. The animal seemed intent on vomiting, but no regurgitation was noted. As the gaping intensified, so did the lizard's respiratory effort, and the animal soon showed severe dyspnea. Within the next 30 min, it underwent a conspicuous color transformation, its dorsal trunk and nape changing from the usual light tan to black. Within the hour after ingestion of the fireflies, and before veterinary assistance could be enlisted, the lizard died.

Postmortem examination showed the animal to have been in good nutritional condition. The stomach contained insect remains (mostly *Acheta* crickets,

a staple food of pet lizards), including body parts of nine *Photinus pyralis*. There were no gross anatomical lesions in stomach, spleen, trachea, heart, lungs, skeletal muscle, kidneys, or brain.

Case 2. The owner of a 7-month-old male *Pogona* sp. (probably *P. vitticeps*) caught a single firefly (evening, mid-summer, Bronx, New York) and introduced it to the lizard's aquarium. The lizard immediately ate the firefly and within 60-90 min began showing oral gaping movements. These became more frequent over the next 30 min, but there was no vomiting. The animal also underwent a color change, from tan to black, in the ventral region of the neck and abdomen, and the back of the tail. In the course of gaping, the lizard tended to protrude and bite its tongue. It then became quiescent and died. Postmortem examination revealed no gross internal lesions. The stomach contents were not scrutinized and the ingested firefly remained unidentified.

RESULTS AND DISCUSSION

There seems little doubt that the two *Pogona* died as a consequence of firefly ingestion. Moreover, in case 1, the firefly, *P. pyralis*, was of a species that we ourselves showed to contain lucibufagins (on the order of 90 μg per individual) (Meinwald et al., 1979; Goetz et al., 1981). In case 2, the firefly was in all likelihood also a *Photinus*, since two species of the genus (*P. ignitus* and *P. marginellus*), both known to contain lucibufagins (on average 60 μg per individual), are abundant in New York State (Eisner et al., 1997). Even if the ingested firefly had been of another genus, say *Photuris* (which also occurs in New York State), the *Pogona* could still have taken in lucibufagins, since female *Photuris* routinely acquire the chemicals by feeding on *Photinus* (Eisner et al., 1997).

Given the lack of detailed information on the toxicity of lucibufagins, little can be said about how the chemicals effected their lethal action on *Pogona*. Cardenolides and bufodienolides are, of course, potently cardiotonic (Budavari et al., 1996), and even at low concentrations they are prone to induce nausea and emesis (Kaiser and Michl, 1958; Kelly and Smith, 1996). Moreover, they can be lethal at remarkably low dosages (ouabain: LD_{50}, intravenous, cat = 0.11 mg/kg; bufalin: LD_{50}, intravenous, cat = 0.14 mg/kg) (Harborne and Baxter, 1993; Kaiser and Michl, 1958). If lucibufagins are comparably toxic and if lizards are as sensitive as cats, a systemic dose of 10–20 μg lucibufagin— less than half the amount in a single *Photinus*—could be lethal to a 100-g *Pogona*. This estimate may not be out of line. Pharmacological tests (by Schering-Plough Corporation) established that lucibufagins induced ventricular arrhythmia in dogs when administered intravenously at a dosage of 0.06 mg/kg. The dose equivalent for a 100-g *Pogona* (assuming again comparable sensitivities) would be the amount of lucibufagin in one tenth of a *Photinus*. Obviously, it would make sense to evaluate the toxicity of lucibufagins

directly, by injecting the chemicals into *Pogona*, but we are unwilling to undertake tests that risk killing these beautiful animals.

It may seem surprising that *Pogona* did not reject *Photinus* on the basis of taste. In our experience with captive vertebrate predators, including a number of mice, birds, amphibians, and *Anolis* lizards, ingestion of potentially lethal insects occurs relatively rarely. Insect defenses, after all, are fashioned typically to take effect before rather than after ingestion. Interesting in this connection is that lizards (*Anolis carolinensis*, *Sceloporus undulatus*, *Eumeces laticeps*) that are sympatric with *Photinus* in southeastern United States reject these insects (Lloyd, 1973; Sexton, 1960, 1964; Sydow and Lloyd, 1975). Could *Pogona*'s failure to reject *Photinus* be a consequence of lack of historical coexistence of these lizards, in their native Australian habitats, with fireflies, or at least with lucibufagin-containing fireflies? Perhaps *Pogona* simply have been spared the selective pressure for evolving an oral aversion to (or systemic tolerance of) lucibufagins. To our knowledge, Australian fireflies have not been studied chemically.

Alternatively, it is conceivable that caged *Pogona*, as a consequence of prolonged confinement or captive breeding, have become "reckless" in their feeding habits. We are disinclined to believe this and feel instead that *Pogona* are naturally incautious. In tests with captive *P. vitticeps* (R. Glor, unpublished), we found these lizards to be aggressively indiscriminate. They readily ate the quinone-spraying cockroach *Diploptera punctata* (Roth and Alsop, 1978), as well as the pyrrolizidine alkaloid-laden moth *Utetheisa ornatrix* (Eisner and Meinwald, 1995). They rejected only bombardier beetles (whose quinonoid spray is hot) (Aneshansley et al., 1969) when first taking these into the mouth, but through persistent assault came to eat even these insects. We used several *P. vitticeps* in these tests and found none to be harmed by ingestion of these insects.

We conclude that *Pogona* pet owners would do well to exercise supervision over the insect diet of their pets, lest they risk losing them. Fireflies, of course, should be altogether excluded from the diet, but so probably should the complex of insects that sequester cardenolides from milkweed plants [including, among others, the monarch and queen butterflies (*Danaus plexippus*, *D. gillipus*), and the lygaeid bug, *Oncopeltus fasciatus*] (Blum, 1981).

Also worth noting is that exotic lizards other than *Pogona* may be susceptible to *Photinus* toxicosis as well. We were informed recently by the owner of several African chameleons (*Chamaeleo pardalis*) in Peoria, Illinois, that one individual (female) died following ingestion of "five or six" fireflies (unidentified).

POSTSCRIPT

Since writing the above we have learned of a case of firefly toxicosis involving White's tree frog, *Litoria* (or *Pelodryas*) *caerulea* (Hylidae), a native of

northern and eastern Australia. Two juvenile frogs ate about three fireflies at about 20 : 00 hr that had been collected near Cleveland, Ohio. The insects continued to flash even after ingestion, as could be seen through the frog's body wall. The frogs were dead the next morning; they did not attempt to regurgitate nor were there signs in the aquarium that they had attempted to do so. They did not change color. We are grateful to Lynn and Martin Rosenberg (Department of Biology, Case Western University, Cleveland, Ohio 44106) for these observations.

More recently, we have learned that a healthy adult specimen of *Lacerta derjugini* (Lacertidae), a native of the Caucasus, died following ingestion of a single firefly (unidentified) that had been collected on a late June day on Long Island, New York. Five minutes after eating the firefly, the lizard began to gape and regurgitated the dead prey; it then rolled on the substrate for 2- 3 min, continued to gape, and was dead within 15 min of consumption. We thank Edward Mariotti (Handex Environmental, Inc., New York) for this information. It seems that firefly toxicosis may be a widespread phenomenon.

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AN ALTERNATE ROUTE TO INSECT PHARMACOPHAGY: THE LOOSE RECEPTOR HYPOTHESIS

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Abstract—The phagostimulatory response of some diabroticite cucumber beetles toward triterpene cucurbitacins is used as a model in support of an alternative hypothesis explaining the evolution of pharmacophagous feeding behavior in insects. Whereas the use of noxious compounds from nonhost sources for purposes other than nutrition or host-plant recognition (pharmacophagy) has historically been explained in terms of the ancestral host hypothesis, we suggest that the less than perfect specificity of the binding properties of some peripheral receptors provides an opportunity for novel compounds sharing the configuration and polarity of target molecules to elicit a feeding response by coincidence rather than adaptive design.

Key Words—Cucurbitacins, pharmacophagy, phagostimulant, pollen, phytosterols, peripheral taste perception, gustation, chemoreception.

INTRODUCTION

Boppré (1984) defined pharmacophagous feeding behavior in insect herbivores as the acquisition of plant-derived compounds for purposes other than primary metabolism or host-plant recognition. Two examples of pharmacophagy have dominated the literature on this subject: the response of certain Blattaria (Blattellidae, Blattidae), Orthoptera (Tettigoniidae, Gryllidae, Pyrgomorphidae),

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Coleoptera (Chrysomelidae, Cerambycidae), Lepidoptera (Danaidae, Ctenuchidae, Riodinidae, Pericopidae, Ithomiidae, Arctiidae, Noctuidae), and Diptera (Tephritidae, Chloropidae) to noxious pyrrolizidine alkaloids (Meinwald et al., 1969; Pliske, 1975; Schneider et al., 1982; Boppré et al., 1984; Krasnoff and Dussourd, 1989; Boppré, 1990), and the behavior of several luperine leaf beetles (Chrysomelidae; Galerucinae, Luperini) toward cucurbitacins, the nonvolatile, bitter tetracyclic triterpenes produced by all members of the Cucurbitaceae (Metcalf et al., 1980; Nishida and Fukami, 1990; Metcalf, 1994). Pharmacophagy of pyrrolizidine alkaloids and cucurbitacins imparts defensive benefits to consumers against predators (Boppré, 1984; Ferguson and Metcalf, 1985; Nishida and Fukami, 1990) and/or pathogens (Tallamy et al., 1998), and has also become an integral component of the reproductive behavior of participating species (Dussourd et al., 1991; LaMunyon and Eisner, 1993; Tallamy et al., unpublished data). In both cases, the pharmacophagous agent is consumed directly by females and/or is sequestered by males and passed within spermatophores to females. Females, in turn, shunt the majority of these materials to developing eggs.

If defense and mating advantages are benefits imparted to all pharmacophagous insects, the selective maintenance of such behavior is no mystery. The origins of insect pharmacophagy, however, are controversial and poorly understood. Although larval host plants of pharmacophagous taxa, by definition, do not produce the chemical that is subsequently sought by adults (Boppré, 1984), larvae of a few ithomiid and danaid relatives of pharmacophagous species develop on plants containing pyrrolizidine alkaloids (Edgar et al., 1974). Thus, students of pyrrolizidine alkaloid pharmacophagy have cautiously concluded that, at some point in the evolutionary history of the diverse group of insects exhibiting this behavior, immatures must have developed on plants that contained the pharmacophagous compound (Pliske, 1975; Boppré, 1978; Edgar, 1982; Trigo and Molta, 1990). This concept was modified by Dussourd (1986) to suggest that variation in the host's production of the compound selected for receptors that enabled adults with inadequate supplies of alkaloids to supplement their needs through pharmacophagous forays to nonhost sources.

The evolution of cucurbitacin pharmacophagy in the Luperini has been viewed similarly (Metcalf, 1979, 1994; Metcalf et al., 1980). Because (1) cucurbitacins are phagostimulants for at least some species of both Old and New World Luperini, (2) at least two genera of Luperini are larval host specialists on cucurbits (Wilcox, 1972; Monroe and Smith, 1980), and (3) all pharmacophagous species tested to date are most stimulated by cucurbitacin B, the most ubiquitous of the 46-plus known cucurbitacin configurations (Hill et al., 1991), luperine responses to cucurbitacins are thought to be plesiomorphic, monophyletic traits that arose from an ancestral host relationship with the Cucurbitaceae. Under this hypothesis, the phagostimulatory response to cucurbitacins of species that currently develop only on noncucurbitaceous host plants [many dozens if not hundreds of species of Dia-

broticites, Cerotomites, Phylecthrites, and Trachyscelites (Wilcox, 1972)] is a relic of a long-lost need for host recognition and is a trait that is currently maintained through secondary selection from protection benefits associated with cucurbitacin consumption (Ferguson and Metcalf, 1985; Tallamy et al., 1998).

The intuitive appeal of the "ancestral host" hypothesis (our label) is obvious, but support for this hypothesis either awaits the construction of appropriate phylogenies, is equivocal because of contradictory phylogenies, or is based upon a paucity of information regarding feeding behavior and host plant affinities. Regardless of whether future phylogenies support or refute the ancestral host hypothesis for insects associated with pyrrolizidine alkaloids or cucurbitacins, recent evidence from other pharmacophagous insects suggests that an ancestral association with a particular compound may not be necessary to promote the evolution of pharmacophagy. Studies of cantharidin, a noxious, volatile monoterpene produced de novo as a nuptial gift exclusively by male meloid and oedomerid beetles, provide a number of examples whereby exposure to a novel and potentially deleterious compound resulted in phagostimulation rather than deterrence. Cantharidin attracts and stimulates feeding in several diverse taxa, including pyrochroid, endomychid, anthicid, staphylinid, and chrysomelid beetles; ceratopogonid, sciarid, and anthomyiid flies; braconid wasps; and mirid and tingid bugs (Young, 1984; Frenzel et al., 1992; Frenzel and Dettner, 1994; Mafra-Neto and Jolivet, 1994; Eisner et al., 1996). If canthariphilous staphylinids, ceratopogonids, mirids, and braconids are truly predaceous or parasitic on meloids and/or fungivorous insects as reported (Young, 1984), their response to cantharidin is merely host-seeking behavior and as such removes them from the ranks of true pharmacophagous insects altogether.

How do we explain, then, a positive response to cantharidin in species that do not prey upon cantharidin-producing beetles? Like meloids and oedomerids, male pyrochroid and anthicid beetles use cantharidin as a nuptial gift with which to procure matings (Schütz and Dettner, 1992; Eisner et al., 1996). Unlike meloids and oedomerids, however, pyrochroids and anthicids cannot produce cantharidin themselves and must consume environmental sources of this compound. Although the appearance of pyrochroids and anthicids at cantharidin baits is no surprise, their use of cantharidin as a nuptial gift is. There are no known environmental sources of cantharidin other than meloid or oedomerid beetles, and pyrochroid and anthicid males are not predaceous on either (Eisner et al., 1996). However, both taxa, as well as the endomychids, sciarids, ceratopogonids, and anthomyiids attracted to cantharidin do feed as larvae and/or adults on fungi (Borror and White, 1970; White, 1983). Canthariphilous staphylinid beetles are predators and, like many staphylinids, may specialize on fungivores (Young, 1984). In fact, the fungivorous habits of many species pharmacophagous toward cantharidin may provide a clue to the origins of this curious behavior. We suggest that early responses to cantharidin could have been stimulated by a similarity in the configu-

ration and polarity of cantharidin to one or more fungal monoterpenes for which fungivorous taxa had evolved specific chemoreceptors. That is, the behavior of at least some canthariphilous insects may have arisen because of the coincidental acceptance of cantharidin as a novel agonist by peripheral receptors with less than perfect specificity rather than from adaptive responses to an historically familiar compound.

The neurophysiological basis of peripheral perception is extraordinarily complex in insect gustatory systems (Frazier, 1986; Simmonds et al., 1990; Schoonhoven et al., 1992; Städler, 1992; Mullin et al., 1994). In the simplest terms, feeding behavior is stimulated if the chemoreception of phagostimulants exceeds the chemoreception of feeding deterrents (Dethier, 1980). In caterpillars and possibly all insects, taste sensilla contain cells specialized for the production of either deterrent and stimulatory inputs, or, more likely, neurons capable of producing both deterrent and stimulatory inputs (Frazier, 1986). Receptor sites on these cells can be highly specific (tight) or less specific (loose). Strychnine, for example, is a compound novel to most phytophagous insects, but it readily depolarizes activation channels leading to deterrent input in most insects; the binding requirements at these sites are sufficiently loose that a variety of molecular structures meet the polarity and configuration specifications for binding there. The loose characteristics of receptor sites with deterrent capabilities may be adaptive because they protect the central nervous system from exposure to damaging novel compounds (Frazier, 1992).

Critical to our argument is the fact that relatively loose binding properties of receptor sites can also enable novel and sometimes deleterious compounds to trigger feeding behavior. There are several mechanisms by which this can happen (Frazier, 1986, 1992). Some molecules bind at receptor sites leading to deterrent inputs, but rather than depolarizing the activation channels, they simply block them. Without inhibitory inputs, even small amounts of phagostimulants, including amino acids present in the insects' saliva, are sufficient to activate the stimulatory inputs at the sensillum and elicit feeding. Activation leading to deterrent inputs can also be inhibited when particular molecules block the stimulus removal system. Finally, loose stimulatory receptor sites themselves can encourage phagostimulation by novel compounds with the appropriate configuration and polarity at binding sites. This is apparently the mechanism by which canthariphilous *Atrichopogon* flies (Ceratopogonidae) respond to terpenes in which the heptane skeleton is associated with either a 2,3-dicarboxylic anhydride or a 2,3-γ-lactone (Frenzel et al., 1992) and by which the peptide aspartame mimics the carbohydrate sucrose at vertebrate receptors, a mimicry upon which much of the sweetener industry is based. We emphasize that considerable variability in response is the rule rather than the exception in insect chemosensory systems (Frazier, 1992). If this variability is even partly genetic, a typical insect population is fertile ground for the advent of novel feeding preferences.

There are numerous examples of inappropriate feeding responses by insects that are presumably the result of imprecision at gustatory receptors. When presented with petunia plants, *Manduca sexta* caterpillars voraciously eat the leaves, pausing only to regurgitate everything they have just eaten. This behavior may continue until the larvae starve to death (Dethier and Crnjar, 1982). Several haustellate arthropods are stimulated to eat in the presence of toxic cucurbitacins. *Tetranychus urticae*, the two-spotted spider mite, prefers cucurbitacin-rich cucumber lines over cultivars without cucurbitacins, even though such behavior reduces mite fitness (Gould, 1978). Similarly, corn delphacids (*Pereqrinus maidis*), sycamore lace bugs (*Corythucha ciliata*), and pea aphids (*Acyrthosiphon pisum*) are all stimulated to feed by exogenous coatings of cucurbitacin B, an evolutionarily novel compound to these species (Tallamy et al., 1997). Mafra-Neto and Jolivet (1994) report seven species of lace bugs (Tingidae) and plant bugs (Miridae), and one luperine chrysomelid beetle, *Diabrotica angulicollis*, eating the cantharidin-rich hemolymph oozing from the joints of disturbed *Epicauta aterrima*, a large meloid beetle from Brazil. Occasional predation is relatively common among mirid plant bugs, but this is the first report of hematophagy among the phytophagous tingids and *Diabrotica* beetles.

The apparent ease with which loose gustatory receptors can lead to an association with novel plant or animal compounds suggests the need to reevaluate the universality of the ancestral host hypothesis as it relates to the evolution of insect pharmacophagy. In our view, the following scenario describes an alternative route to pharmacophagy that in several cases seems more parsimonious than assumptions about long-lost host relationships. In theory, an insect with gustatory receptors that evolved in the context of meeting a particular suite of nutritional and pharmacological needs could suddenly encounter a novel compound that, for one or more of the reasons discussed above, elicits a feeding response. If the compound enhances the fitness of those that consume it, the response should eventually move to fixation within the population, assuming it is genetically based and encounters with the novel compound are sufficiently frequent. If the molecule is toxic, early consumers will suffer reduced fitness. This will not, however, lead to a tightening of the responsible receptor's specificity unless selection to avoid the new compound exceeds selection to maintain the loose properties of the receptor in question. Pharmacophagy should ensue when: (1) exposure to the novel compound is sufficiently frequent to select for physiological tolerance and (2) the compound imparts distinct mating and/or defensive benefits to its consumers.

To illustrate the loose receptor hypothesis in the context of an actual pharmacophagous relationship we will briefly apply it to luperine cucurbitacin pharmacophagy. Cucurbitacin pharmacophagy is currently assumed to have arisen through ancestral larval relationships with cucurbitacin-producing cucurbits (Metcalf et al., 1980), but recent evidence that cucurbitacin pharmacophagy

is intricately associated with an adult affinity for pollen introduces an alternative possibility: that the phagostimulatory response to cucurbitacins arose through adult feeding behaviors rather than through larval host dependencies. Although data are few, review of the adult feeding habits of luperines suggests that there is a perfect correlation between adult affinity for pollen and cucurbitacin phagostimulation: pollen is a substantial component of the adult diet in species stimulated to eat by cucurbitacins (*Diabrotica*, *Acalymma*, *Aulacophora*), while species in which the adult diet is largely confined to foliage are repelled by cucurbitacins (*Ceratoma trifurcata*, all Luperina) (Metcalf et al., 1980; Sinha and Krishna, 1969; Tallamy, unpublished data).

Amino acid neuroreceptors exhibiting γ-aminobutyric acid (GABA)/glycine pharmacology are located on the maxillary galeae of *Diabrotica* and have been implicated in both the perception of antifeedants and phagostimulants (Mullin et al., 1992; Chyb et al., 1995; Hollister and Mullin, 1998; Kim and Mullin, 1998). Cucurbitacins do not occur in pollen (Andersen and Metcalf, 1987), but pollen and meristematic tissues favored by adult pharmacophagous luperines are enriched with similar mid-polar (mildly lipophilic) compounds such as brassinosteroids, ω-3 linoleic acid-containing lipids, and hydroxycinnamic acid–polyamine amides, along with polar low-molecular-weight neutral amino acids including GABA (Barbier, 1971; Stanley and Linskens, 1974; Erhardt and Baker, 1990; Marquardt and Adam, 1991; Feldlaufer et al., 1993; Mullin et al., 1993; Lin and Mullin, 1999). Many of these compounds elicit phagostimulatory responses from *Diabrotica* amino acid receptors (Mullin et al., 1994; Hollister and Mullin, 1998; Lin and Mullin, 1999), either alone or while interacting at multiple sites. In particular, some pollen sterols potentiate the amino acid agonists in pollen (Chyb et al., 1995; Hollister and Mullin, 1998; Kim and Mullin, 1998). Current data suggest that it is these same taste neurons that are depolarized by cucurbitacins (Mullin et al., 1994).

Cucurbitacins are structurally similar to many sterols, sharing both their hydroxylation and stereochemistry (Dinan et al., 1997a,b). The unusual 4,4-dimethyl-5-ene structure of cucurbitacin B, for example, orients the 3-one group below the ring plane (Mullin et al., 1994) and thus closely resembles GABA-acting 3α-ol pregnane steroids (Purdy et al., 1990). Like pregnane steroids, cucurbitacins potentiate amino acids such as alanine, serine, proline, and GABA (compounds present in pollen and saliva alike) and trigger a feeding response in *Diabrotica* even at very low doses (Mullin et al., 1994; Kim and Mullin, unpublished data). The structural similarity between cucurbitacins and many sterols also renders these molecules powerful antagonists at insect ecdysteroid receptors (Dinan et al., 1997a,b). It is possible that selection on luperine amino acid receptors for the loose perception of pollen constituents simultaneously rendered these receptors susceptible to cucurbitacin depolarization. Since ancestral chrysomeloid beetles were associated with polliniferous food sources even before the

division of the cerambycid and chrysomelid lineages in the early Cretaceous (Samuelson, 1994), it is likely that in certain lineages pollen feeding and the evolution of taste neurons associated with pollen feeding preceded luperine interactions with cucurbits and their cucurbitacins. Thus, the response of Luperini to cucurbitacins may not reflect an ancestral larval association with cucurbitacin-producing plants as suggested for luperines in particular (Metcalf, 1979) and implied by pharmacophagous theory in general (Edgar, 1982). Rather, it may be the product of a physiological coincidence mediated by the molecular configuration of cucurbitacins that promotes binding at a steroidal site on amino acid receptors.

Under this scenario, beetles that evolved to exploit cucurbit pollen because of its abundance and nutritional richness (Reddi and Aluri, 1993) were, regardless of their larval host, serendipitously placed in contact with cucurbitacins, which are components of cucurbit anthers and flower petals, but not pollen (Andersen and Metcalf, 1987). In the early stages of the interaction, beetles that were stimulated to feed on cucurbitacins would have suffered reduced fitness; nevertheless, cucurbitacin avoidance could not have evolved without a significant tightening of the pollen-adapted amino acid receptors, compromising the detection of an evolutionarily entrenched adult food source. Repeated exposure to cucurbitacins would have favored physiological tolerance, while advantages gained from their defensive properties would have encouraged beetle associations with cucurbitacin sources.

We do not suggest that the ancestral host hypothesis is without merit and should be discarded in favor of the loose receptor hypothesis. Clearly the ancestral host hypothesis provides the most parsimonious explanation for the evolution of pharmacophagy whenever an ancestral association with the pharmacophagous compound can be demonstrated. For example, there is some evidence that the Australian ithomiid butterfly *Tellervo zoilus zoilus* Fabricius is a basal taxon in the Ithomiinae (Fox, 1956; Emmel et al., 1974) and that larvae of this species may feed on *Parsonia velutina* (Apocynaceae), a plant containing pyrrolizidine alkaloids (Edgar 1982). If the antiquity of this species and its larval host association is confirmed, the feeding behavior of descendants that are pharmacophagous toward pyrrolizidine alkaloids can be explained by the ancestral host hypothesis. But were all of the ancestors of the hundreds of Lepidoptera taxa (271 spp from 100 genera in eight families from northern South America) recorded by Pliske (1975) at pyrrolizidine alkaloid baits host specialists on plant sources of this compound? And does the ancestral host hypothesis provide the most parsimonious explanation of aberrant meloid hematophagy in phytophagous lace bugs and leaf beetles, or of cantharidin pharmacophagy in a phylogenetically disparate array of insect fungivores, or of pyrrolizidine alkaloid pharmacophagy by several species of generalist cockroach detritivores? We are skeptical and encourage the inclusion of the loose receptor hypothesis in future endeavors to explain the evolution of insect pharmacophagy.

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HOST-PLANT CHEMISTRY INFLUENCES OVIPOSITION CHOICE OF THE SPICEBUSH SWALLOWTAIL BUTTERFLY

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Abstract—The spicebush swallowtail, *Papilio troilus* (L.), lays its eggs on plants in the family Lauraceae. Sassafras [*Sassafras albidum* (Nutt.) Nees], spicebush [*Lindera benzoin* (L.) Spreng.], redbay (*Persea borbonia* (L.)] and camphortree [*Cinnamomum camphora* (Nees) Eberm.] are four of its known host plants. In one-choice tests, free-flying spicebush swallowtail females laid eggs on chemical extracts of the leaves of each of these four hosts. In two-choice experiments, females always preferred to oviposit on an extract of sassafras compared to extracts of the other three hosts. It was shown for spicebush extract that this response was not due to oviposition experience. Previously we had identified one of the host plant chemicals acting as an oviposition stimulant in sassafras extract as 3-caffeoyl-*muco*-quinic acid (3-C*m*QA). Extracts of the other three hosts did not contain this compound. The addition of 3-C*m*QA alone to spicebush extract did not increase oviposition activity. It did, however, increase discrimination between hosts and nonhosts. When a fraction of sassafras extract containing 3-C*m*QA and other synergistic stimulants was added to spicebush extract, preference for sassafras extract was no longer recorded. These results show existing differences in oviposition chemistry among host plants of the spicebush swallowtail and how these differences can influence oviposition choice in bioassay experiments.

Key Words—Oviposition stimulants, Lauraceae, *Sassafras albidum*, *Lindera benzoin*, *Persea borbonia*, *Cinnamonum camphora*, Papilionidae, *Papilio troilus*, 3-caffeoyl-*muco*-quinic acid, host-plant discrimination.

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INTRODUCTION

The spicebush swallowtail, *Papilio troilus* (L.), is a generalized specialist, laying its eggs on five species of plants in four different genera of the family Lauraceae (Lederhouse et al., 1992). This plant family is primarily tropical and subtropical in distribution, with only a few species reaching into the temperate region (Kostermans, 1957). Two of its host plants, sassafras [*Sassafras albidum* (Nutt.) Nees] and spicebush [*Lindera benzoin* (L.) Spreng.], cooccur with the nominate subspecies, *P. troilus troilus*, throughout most of its range in Eastern North America, excluding central and southern Florida (Nitao et al., 1991; Tietz, 1972). There, redbay [*Persea borbonia* (L.)] is the host plant of the southern subspecies, *P. t. ilioneus* (Hagen and Scriber, 1991; Nitao et al., 1991). An additional recorded host plant of *P. t. troilus* is camphortree [*Cinnamomum camphora* (Nees) Eberm.], an ornamental shade tree introduced to the southeast United States from Asia (Morris, 1989; Tietz, 1972). Three other species of swallowtails in the tribe Papilionini, *P. thaiwanus* (I. A. Takashi, personal communication), *Chilasa agestor*, and *C. epycides*, and one species in the tribe Graphiini, *Graphium sarpedon sarpedon* (Linné) (Igarashi, 1979) are reported to use camphortree as a host plant. *P. t. troilus* has also been recorded from silky spicebush, *L. melissifolium* Walt. (Morris, 1989).

Host-plant chemistry plays a major role in contact recognition by swallowtail butterflies (Carter et al., 1998; Feeny, 1987, 1991, 1992, 1995; Haribal et al., 1998; Nishida, 1995; Oshugi, 1991). One chemical acting as an oviposition stimulant for the spicebush swallowtail from the leaves of sassafras has been identified as 3-caffeoyl-*muco*-quinic acid (3-C*m*QA) (Carter et al., 1999). The research presented here reveals differences in behavioral responses of spicebush swallowtail females, *P. t. troilus*, to the chemical extracts of four of its host plants.

METHODS AND MATERIALS

Insects and Bioassay Design. All butterflies were offspring from diapausing pupae originating in either Pennsylvania or Tennessee. Female butterflies used in bioassays had been mated and allowed to oviposit on host-plant leaves. All females had experience ovipositing on the primary host before the start of an experiment. In all experiments except three, females had previous experience only on sassafras leaves immediately preceding and between trials. In the other three experiments (see below: Table 2, experiments 3 and 9, and Table 3, experiment 2), females had experienced ovipositing only on spicebush leaves immediately preceding and between trials. All eight trials of an experiment were completed before a new experiment was initiated. Leaves for oviposition were

presented as potted plants grown in a greenhouse or as cut sprigs of foliage collected in the field. On a test day, host foliage was covered with a net cage in the late morning. Oviposition trials were conducted in early afternoon and lasted approximately three hours. Six to 20 mated female butterflies were present during a trial. Upon completion of a trial, host foliage was made available for butterfly oviposition.

Sassafras and spicebush were the only two hosts with abundant fresh leaf material for bioassay of intact leaves and preparation and bioassay of an alcoholic extract. Host-plant leaves were tested as similar-looking sprigs, each of three alternate leaves of sassafras or spicebush. Each sprig was placed in a water-filled aquapic buried in a pot of soil. Controls were a bean plant with three alternate leaves. To test host-plant extracts the oviposition substrate was a nonhost fava bean plant (Broad Windsor, 10–14 days old). Untreated bean plant leaves were not active in bioassay, yet when they were treated with host-plant extracts we observed reliable butterfly oviposition responses with no deterrent effect. Bean leaves were chosen to have consistently similar leaf areas, not differing by more than 10%. Volumes of plant extracts or fractions and solutions of pure compounds or solvents were transferred into a small (5-ml) TLC reagent applicator by using an HPLC syringe. Plant extracts were tested at 0.05 gle (gram leaf equivalents, wet weight) diluted in 2 ml of 50% MeOH (aq.), and the full dose applied evenly over the upper surfaces of two leaves of a bean plant. The amount (100 μg) of a pure oviposition stimulant, 3-C*m*QA, was shown previously to be active at a dose of 7 ng/mm^2 of bean leaf surface (Carter et al., 1999). For each trial, two experimental plants, treated with test extracts or solutions, and two control plants, treated with the solvents MeOH and water, were alternately placed on the four corners of a table in a growth chamber with free-flying butterflies. Plants were rotated around the table in a clockwise direction every 30 min. At the end of a trial, eggs laid on the host-plant leaves or on the treated bean plant leaves were counted. Eggs from the two control plants were averaged. One-choice experiments had only one treatment and the control presented to the butterflies, while two-choice experiments had two different treatments and the control.

Each experiment was the combined results of eight identical trials. Data were analyzed by a paired t test for the mean proportion of eggs laid on either the treatment plants or the control plants as compared to the expected value of 0.5, assuming the null hypothesis of no oviposition preference between control and treatment. In two-choice tests, the mean proportion of eggs laid on each of the two treatments was compared directly by a paired t test.

Preparation of Plant Extracts. Sassafras and spicebush foliage was collected in Tompkins County, New York. Redbay leaves were collected and shipped from Marion County, Florida. Camphortree leaves were shipped from a greenhouse in California. Leaves were weighed before boiling in 95% EtOH in

a blender for 5 min. Each slurry was filtered over nylon floss, and the particulate matter rinsed three times with hot EtOH. Extracts were reduced by rotary evaporation at <50°C to remove EtOH and centrifuged to remove chlorophyll. The resulting aqueous layers were labeled alcoholic extracts. Post-EtOAc aqueous extracts of each host were prepared by sequential extraction of all four alcoholic extracts with three volumes each of the following organic solvents: hexane, diethylether (Et_2O), chloroform ($CHCl_3$), and ethylacetate (EtOAc). The residual aqueous layers were reduced by rotary evaporation, and the remaining material was dissolved in water, 1–10 gle/ml.

C-18 Column Chromatography of Post-EtOAc Aqueous Extract of Sassafras. To isolate sassafras oviposition stimulants, 25 gle of the post-EtOAc aqueous extract was separated on a flash column (19 × 50 mm) packed with C-18 Bonded Phase (Baker, 40 μm particle size). Elution of the column with 300 ml of 1% acetic acid was followed by elution with 100 ml of 10% MeOH in 1% acetic acid (fraction B). Fraction B was reduced by rotary evaporation and tested for oviposition activity. 3-C*m*QA was isolated from fraction B by column chromatography by using CHP20P (14 × 150 mm; 75–100 μm) and eluted with a gradient of 10% MeOH in 1% acetic acid to 45% MeOH in 1% acetic acid by increasing the proportion of MeOH in 5% increments (Carter et al., 1999).

*HPLC of Post-EtOAc Aqueous Host Plant Extracts, Fraction B and 3-C*m*QA.* The four host-plant post-EtOAc aqueous extracts, fraction B and solutions of 3-C*m*QA and chlorogenic acid (5-CQA) in 50% MeOH (aq.), were chromatographed on a C-18 HPLC column (Columbus, 10 × 250 mm, 5 μm) by using a solvent gradient of 95% of 1% acetic acid in MeOH to 100% MeOH (flow rate 3 ml/min; curve 6) monitored by UV (λ_{254}).

RESULTS

Activity of Intact Leaves and Alcoholic Extracts of Sassafras and Spicebush. In a two-choice test, no significant oviposition preference was recorded between intact sassafras and spicebush leaves (Table 1, experiment 1). When tested against spicebush alcoholic extract, there was a significant oviposition preference for sassafras alcoholic extract (Table 1, experiment 2).

Activity (One-Choice Bioassays) of Post-EtOAc Aqueous Host-Plant Extracts. In one-choice experiments, female spicebush swallowtails laid significantly more eggs on bean plants treated with post-EtOAc aqueous extracts of sassafras, spicebush, redbay, and camphortree than on control plants treated with solvent alone, indicating contact chemical oviposition stimulants were present in all four post-EtOAc aqueous extracts (Table 2, experiments 1, 2, 4, and 5).

Activity of Post-EtOAc Aqueous Extracts of Sassafras and of Other Three

TABLE 1. OVIPOSITION RESPONSES OF FEMALE SPICEBUSH SWALLOWTAIL BUTTERFLIES TO LEAVES AMD ALCOHOLIC EXTRACTS OF SASSAFRAS AND SPICEBUSH[a]

Treatment 1	$\overline{X}$[b]	E[c]	Treatment 2	$\overline{X}$	E	SE[d]	P[e]
Expt. 1 Sassafras leaves vs. spicebush leaves							
Control	0.00	0	Sassafras	1.00	456	0.00	f
Control	0.00	0	Spicebush	1.00	466	0.00	f
Sassafras	0.50	456	Spicebush	.50	466	0.06	NS
Expt. 2 Sassafras alcoholic extract vs. spicebush alcoholic extract (0.05 gle each)							
Control	0.02	45.5	Sassafras	0.98	1317	0.01	0.001
Control	0.03	45.5	Spicebush	0.97	969	0.02	0.001
Sassafras	0.59	1317	Spicebush	0.41	969	0.02	0.006

[a]Each experiment combined the results of eight replicated trials (N = 6–20 females/trial; NS = not significant, $P \geq 0.05$).
[b]Mean ($\overline{X}$) proportion of eggs laid on each of the two treatments in a pair.
[c]Total number of eggs laid per treatment.
[d]Standard error of the means (identical for both treatments in each pair of proportional data).
[e]Probability that the mean number of eggs laid between a pair does not differ from the null hypothesis of 0.5.
[f]No statistics.

Hosts. In two-choice experiments, female spicebush swallowtails always preferred sassafras post-EtOAc aqueous extract to post-EtOAc aqueous extracts of the other three hosts (Table 2, experiments 6–9). All four extracts were active when compared to the control plants in the same experiment (Table 2, experiments 6–9). Females with experience ovipositing only on spicebush leaves also preferred sassafras extract (Table 2, experiment 9), suggesting oviposition results in experiments 6–9 were not significantly influenced by pretest oviposition experience.

Analysis of Host-Plant Post-EtOAc Aqueous Extracts. HPLC analysis of post-EtOAc aqueous sassafras extract showed a large UV absorbing peak (λ_{254} max. 325.9; 235.7) at a retention time (R_t) of 24.9 min, previously identified as 3-C*m*QA (Carter et al., 1999). This compound matched the R_t and UV spectrum of a solution of 3-C*m*QA purified from sassafras post-EtOAc aqueous extract found in fraction B. A detailed examination of the HPLC peaks in this same chromatographic region of the post-EtOAc aqueous extracts of the other three hosts did not reveal any peaks matching the UV spectrum and R_t of this compound. 5-CQA, an oviposition stimulant for *P. polyxenes* (Feeny et al., 1988) and *P. protenor* (Honda, 1990), was not present in the extracts of sassafras, spicebush, and camphortree. There was a peak corresponding in R_t (38.9 min) and UV spectrum (λ_{254} max. 325.9; 240.7) to 5-CQA in redbay extract.

Activity of Spicebush Post-EtOAc Aqueous Extract Combined with 3-CmQA or with Fraction B from Sassafras Post-EtOAc Aqueous Extract. When 3-C*m*QA alone was added to spicebush post-EtOAc aqueous extract, this was still less

TABLE 2. OVIPOSITION RESPONSES OF FEMALE SPICEBUSH SWALLOWTAIL BUTTERFLIES TO POST-EtOAc AQUEOUS EXTRACTS OF SASSAFRAS, SPICEBUSH, SPICEBUSH + 3-C*m*QA, CAMPHORTREE, AND REDBAY ALONE AND POST-EtOAc AQUEOUS EXTRACT OF SASSAFRAS COMPARED TO POST-EtOAc AQUEOUS EXTRACTS OF SPICEBUSH, REDBAY, AND CAMPHORTREE[a]

Treatment 1	$\overline{X}$[b]	E[c]	Treatment 2	$\overline{X}$	E	SE[d]	P[e]
Expt. 1. Post-EtOAc extract of sassafras leaves (0.05 gle)							
Control	0.02	19	Sassafras	0.98	1140	0.01	0.001
Expt. 2. Post-EtOAc extract of spicebush leaves (0.05 gle)							
Control	0.20	40	Spicebush	0.80	169	0.02	0.001
Expt. 3[f] Post-EtOAc extract of spicebush leaves (0.05 gle) + 3C*m*QA (100 μg)							
Control	0.12	26	Spicebush + 3-C*m*QA	0.88	228	0.02	0.001
Expt. 4. Post-EtOAc extract of redbay leaves (0.05 gle)							
Control	0.09	22	Redbay	0.91	358	0.04	0.001
Expt. 5. Post-EtOAc extract of camphortree leaves (0.05 gle)							
Control	0.02	5	Camphortree	0.98	248	0.01	0.001
Expt. 6. Sassafras post-EtOAc vs. spicebush post-EtOAc (0.05 gle each)							
Control	0.19	19	Spicebush	0.81	219	0.08	0.001
Control	0.01	19	Sassafras	0.99	1795	0.03	0.001
Spicebush	0.10	219	Sassafras	0.90	1795	0.02	0.001
Expt. 7. Sassafras post-EtOAc vs. redbay post-EtOAc (0.05 gle each)							
Control	0.10	57	Redbay	0.90	797	0.02	0.001
Control	0.03	57	Sassafras	0.97	1888	0.01	0.001
Redbay	0.30	797	Sassafras	0.70	1888	0.06	0.001
Expt. 8 Sassafras post-EtOAc vs. camphortree post-EtOAc (0.05 gle each)							
Control	0.07	23.5	Camphortree	0.93	327	0.02	0.001
Control	0.01	23.5	Sassafras	0.99	1793	0.01	0.001
Camphortree	0.15	327	Sassafras	0.85	1793	0.03	0.001
Expt. 9[f] Spicebush post-EtOAc vs. sassafras post-EtOAc (0.05 gle each)							
Control	0.01	69	Sassafras	0.99	2453	0.01	0.001
Control	0.01	69	Spicebush	0.89	598	0.04	0.001
Spicebush	0.20	598	Sassafras	0.80	2453	0.03	0.001

[a]Each experiment combined the results of 8 replicated trials (N = 6–20 females/trial; NS = not significant, $P \geq 0.05$).
[b]Mean ($\overline{X}$) proportion of eggs laid on each of the two treatments in a pair.
[c]Total number of eggs laid per treatment.
[d]Standard error of the means (identical for both treatments in each pair of proportional data).
[e]Probability that the mean number of eggs laid between a pair does not differ from the null hypothesis of 0.5.
[f]Female butterflies had oviposition experience on only spicebush leaves both before and during this experiment.

TABLE 3. OVIPOSITION RESPONSES OF FEMALE SPICEBUSH SWALLOWTAIL BUTTERFLIES TO SPICEBUSH POST-EtOAc AQUEOUS EXTRACT + 3-CmQA OR FRACTION B FROM SASSAFRAS[a]

Treatment 1	$\overline{X}$[b]	E[c]	Treatment 2	$\overline{X}$	E	SE[d]	P[e]
Expt. 1. Sassafras post-EtOAc (0.05 gle) vs. spicebush post-EtOAc (0.05 gle) + 3-CmQA (100 μg)							
Control	0.01	30.5	Sassafras	0.99	2630	0.01	0.001
Control	0.08	30.5	Spicebush + 3-CmQA	0.92	397	0.02	0.001
Sassafras	0.86	2630	Spicebush + 3-CmQA	0.14	397	0.03	0.001
Expt. 2.[f] Spicebush post-EtOAc (0.05 gle) vs. spicebush post-EtOAc (0.05 gle) + 3-CmQA (100 μg)							
Control	0.03	13.5	Spicebush	0.97	207	0.02	NS
Control	0.05	13.5	Spicebush + 3-CmQA	0.95	214	0.02	NS
Spicebush	0.55	207	Spicebush + 3-CmQA	0.45	214	0.07	NS
Expt. 3. Fraction B from sassafras (0.05 gle)							
Control	0.07	47	Fr. B	0.93	628	0.01	0.001
Expt. 4. Fraction B vs. Sassafras post-EtOAc (0.05 gle each)							
Control	0.04	11	Fr. B	0.96	277	0.02	0.001
Control	0.01	11	Sassafras	0.99	1916	0.01	0.001
Fr. B	0.15	277	Sassafras	0.85	1916	0.04	0.001
Expt. 5. Sassafras post-EtOAc vs. spicebush post-EtOAc + fraction B (0.05 gle each)							
Control	0.03	20	Sassafras	0.97	732	0.01	0.001
Control	0.03	19	Spicebush + Fr. B	0.97	736	0.01	0.001
Sassafras	0.51	732	Spicebush + Fr. B	0.49	736	0.02	NS

[a]Each experiment combined the results of 8 replicated trials ($N = 6$–20 females/trial; NS = not significant, $P \leq 0.05$).
[b]Mean ($\overline{X}$) proportion of eggs laid on each of the two treatments in a pair.
[c]Total number of eggs laid per treatment.
[d]Standard error of the means (identical for both treatments in each pair of proportional data).
[e]Probability that the mean number of eggs laid between a pair does not differ from the null hypothesis of 0.5.
[f]Female butterflies experienced ovipositing on only spicebush leaves before and during this experiment.

active than sassafras post-EtOAc aqueous extract (Table 3, experiment 1). The combination of spicebush post-EtOAc aqueous extract plus 3-CmQA did not differ from spicebush post-EtOAc aqueous extract alone (Table 3, experiment 2). This indicated that spicebush extract did not contain the unidentified synergistic compound(s) needed to stimulate an oviposition response to 3-CmQA (Carter et al., 1999).

From column chromatography, fraction B of sassafras post-EtOAc aqueous extract containing 3-CmQA plus the other synergistic oviposition stimulants was isolated and shown to be active in bioassay (Table 3, experiment 3). However, it was significantly less active than sassafras post-EtOAc aqueous extract (Table 3, experiment 4). In a two-choice experiment, the combination of spicebush post-EtOAc aqueous extract plus fraction B from sassafras was not significantly different in eliciting oviposition activity from sassafras post-EtOAc aqueous extract (Table 3, experiment 5).

Differences in Proportion of Eggs Laid on Control Plants for Three Treatments of Sassafras, Spicebush, and Spicebush plus 3-CmQA Post-EtOAc Aqueous Plant Extracts. Trials of the individual post-EtOAc aqueous extracts of sassafras, spicebush, and spicebush plus 3-C*m*QA showed differences in the proportions of eggs laid on control plants (Friedman's test, $\chi^2 = 14.25$, $P \leq 0.05$). A smaller proportion of eggs (Wilcoxon sign rank test, $T_s = 0$, $P \leq 0.01$) were laid on controls in the presence of sassafras extract (0.02; Table 2, experiment 1) compared to the proportion of eggs laid on controls in the presence of spicebush extract (0.20; Table 2, experiment 2). Addition of 3-C*m*QA to spicebush extract significantly ($T_s = 5$, $P \leq 0.05$) decreased the proportion of eggs laid on control plants (0.12, Table 2, experiment 3) compared to the proportion of eggs laid on controls in the presence of spicebush extract alone. The proportion of eggs on control plants in the presence of spicebush extract plus 3-C*m*QA was still ($T_s = 0$, $P \leq 0.01$) larger than the proportion of eggs on controls while testing sassafras extract.

DISCUSSION

Leaves of sassafras, spicebush, redbay, and camphortree stimulate egg-laying by the spicebush swallowtail (Carter and Feeny, unpublished data). We could find no preference between leaves of sassafras or spicebush. Similar results were found by Lederhouse et al. (1992), who tested oviposition preference of spicebush swallowtail females for host foliage of sassafras, spicebush, and redbay in plastic box experiments. While sassafras and spicebush foliage received equivalent proportions of eggs (43% and 46%), comparable to our results, redbay was the least preferred (11%) (Lederhouse et al., 1992).

In contrast to the responses to intact foliage, an ethanolic extract of sassafras was more active than an equivalent spicebush extract. During preparation and testing of the ethanolic extracts, we removed host-specific visual and olfactory cues that may have contributed to the full activity of intact spicebush leaves. Such cues are known to be important for other swallowtail species (Baur and Feeny, 1992; Baur et al., 1993; Papaj, 1986a; Rausher, 1978; Feeny et al., 1989). As we purified the extracts further to the post-EtOAc aqueous stage, removing the less-polar compounds, preference for sassafras extract increased. This suggests that in spicebush leaves less polar compounds acting as contact oviposition stimulants are present. This contrasts with previous research on sassafras extract in which less polar compounds were found not to increase the oviposition response to polar contact stimulants (Carter et al., 1999).

Spicebush, redbay, and camphortree extracts appear to lack 3-C*m*QA as well as (in spicebush, at least) additional, as yet unidentified, contact oviposition stimulant(s) found in fraction B. Contact oviposition chemistry clearly is not identi-

cal for all four hosts. The addition of fraction B, containing both 3-C*m*QA and the other stimulant(s), resulted in no difference in oviposition response between sassafras and spicebush post-EtOAc aqueous extracts. This result suggests that these stimulants are the key factors mediating the oviposition preference of sassafras post-EtOAc aqueous extract. It also suggests chemical oviposition deterrents were not responsible for this difference, as activity was fully restored with the addition of fraction B to spicebush post-EtOAc aqueous extract. We know (Carter and Feeny, unpublished) that, at least for sassafras, additional classes of polar compounds other than cinnamic acid derivatives are involved in host-plant recognition. Both *P. polyxenes* (Feeny et al., 1988; Carter et al., 1999) and *P. protenor* (Honda, 1990) can recognize different host plants by using unique profiles or different combinations of chemical contact stimulants. The spicebush swallowtail does not oviposit only in response to a rigid template of contact oviposition stimulants. It is possible, however, that a critical ingredient is common to all four post-EtOAc aqueous host-plant extracts that enhances oviposition.

Addition of 3-C*m*QA to spicebush extract may have increased egg-laying discrimination between treatment and control plants, resulting in fewer eggs, or oviposition mistakes, on control plants. There was evidently sensory recognition of 3-C*m*QA even in the absence of other compound(s) needed to increase oviposition responses. Host-plant discrimination has been studied extensively in the pipevine swallowtail butterfly, *Battus philenor* (Papaj, 1986b; Rausher, 1995), which also uses primarily chemotactile cues during postalighting oviposition behavior to distinguish between hosts and nonhosts. In general, discrimination between treatments and controls was best when testing intact host-plant leaves (proportion of eggs on control plants was 0%), suggesting additive effects between polar contact oviposition chemicals and other sensory cues that influence host-plant discrimination.

Our findings confirm earlier results (Honda, 1990; Carter et al., 1999) suggesting that postalighting recognition of different host plants by swallowtail females in the genus *Papilio* is mediated not by a single fixed profile of contact stimulants but rather by a range of possible profiles corresponding to differences in plant chemistry. In the case of *P. troilus*, at least, the extent to which such profiles share particular key stimulant compounds has yet to be established. This is in contrast to swallowtails in the tribe Troidini, where one or more isomers of aristolochic acid are required in the oviposition profiles studied so far (Nishida, 1995). Flexibility in the use of visual and volatile cues, and chemical contact cues from primarily ubiquitous classes of compounds for oviposition recognition of host plants (Nishida, 1995) has allowed for successful worldwide radiation and adaptation of swallowtail butterflies (Feeny, 1995).

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THE EFFECT OF ATMOSPHERIC PHEROMONE CONCENTRATIONS ON BEHAVIOR OF LIGHTBROWN APPLE MOTH IN AN APPLE ORCHARD

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Abstract—A previously validated Lagrangian model was used to estimate the threshold of atmospheric pheromone concentration required to prevent trap catch and wing fanning in mating disruption plots in an apple orchard. Electroantennogram (EAG) traces of 10 min duration were recorded, along with supporting meteorological data needed for the model, to better define the conditions in which successful mating disruption will occur. Pheromone was released from polyethylene tubing dispensers into orchard blocks treated with 10, 100, 1000, and 2000 dispensers/ha. Predicted dusk concentrations of atmospheric pheromone at a height of 1.85 m varied nightly between 4 and 90 ng pheromone/m^3 (in plots treated with 1000 dispensers/ha) over 11 weeks. Disruption of traps baited with 1000-μg pheromone lures followed an asymptotic curve with predicted concentration, but they did not show a significant effect of trap height (1.5 and 3.0 m). Wing fanning was reduced by increasing the density of dispensers, but was not completely eliminated even at 1000 dispensers/ha. At this density, the concentrations were usually <16 ng pheromone/m^3. Electroantennogram recordings of 10 min duration showed a higher frequency of pheromone pulses in plots treated with more point sources per hectare. There was also a positive correlation between the number of pulses recorded by the EAG and predicted concentration for plots treated with 1000 or 2000 dispensers/ha. These results give added support to our model of pheromone release and transport in treated apple orchards.

Key Words—*Epiphyas postvittana*, Tortricidae, sex pheromone, mating disruption, threshold concentration, behavior, model, wind.

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INTRODUCTION

The conditions required for successful mating disruption in treated apple orchards and the mechanisms involved appear to vary among insect species. Potential mechanisms have been reviewed in Bartell (1982), Cardé (1990), and Sanders (1996) and have a bearing on the design of the optimal pheromone blend, as well as the delivery system. It is generally agreed that mating disruption requires a sufficient concentration of pheromone over a defined time period (time–dose product) in the atmosphere of the treated crop.

There is only a poor understanding, however, of the atmospheric concentrations and plume structure of pheromone that lead to successful disruption in the field. This is mainly due to technical difficulties in measuring the low concentrations involved. Only a few attempts have been made at measurement of atmospheric pheromone concentrations, such as in treated cotton fields (Caro et al., 1980; Flint et al., 1990), pea fields (Witzgall et al., 1996), tea plantations (Uchijima, 1988), and in Japanese apple orchards (Ogawa, personal communication). Measurements were made over several hours on a relatively small number of occasions, which may or may not have been truly representative.

Modeling of atmospheric pheromone concentrations is one solution to overcome the technical difficulties encountered with direct measurement of pheromone, which is normally only a few parts per billion (Suckling et al., 1999). Modeling should permit an examination of disruption under a range of environmental and operational conditions. Gaussian "plume" models (e.g., Sutton, 1953; Wright, 1958) have been used to predict pheromone concentrations and distributions downwind from a single point source. However, the pheromone concentration and the temporal pattern (plume structure) perceived by an insect downwind is different from that predicted by such time-averaged models. Furthermore, they are not useful for predicting concentrations in orchards, since the scalar transport phenomena are different from those in the open air (Raupach, 1988). In orchards, the frequent up- and down-drafts of wind responsible for the rapid transport correlate with rapid changes in atmospheric concentrations of pheromone (Suckling and Karg, 1997a). Suckling et al. (1999) reported the validation of a more appropriate Lagrangian model to predict concentrations in treated orchards. This model is based on the statistical properties of turbulent airflow in the canopy and was found to be within a factor of two of the measured values of pheromone for a range of dispenser densities and weather conditions. This model requires input of the release rate from the dispensers. Here, we use polyethylene dispensers, which can be modeled (Brown et al., 1992; Bradley et al., 1995). For example, with dispensers containing stable formulations such as tetradecenyl acetates, the instantaneous release rates can be estimated from temperature and liquid length information (Bradley et al., 1995). The Lagrangian model also takes into account parameters such as the orchard canopy (tree height

and distribution of foliage), the pheromone dispensers (release rate, number, and vertical spread of dispensers), and meteorological conditions (air temperature, windspeed at the tree top). These parameters are used to predict vertical profiles of pheromone concentrations in orchards (Suckling et al., 1999). Application of the Lagrangian model may highlight ways to improve mating disruption, for example, by demonstrating the optimum placement of dispensers in the field.

Field electroantennogram (EAG) recordings also have been made for the purpose of indicating pheromone levels in orchards (Karg et al., 1994; Suckling et al., 1994). Interaction of pheromone and plant volatile perception has made the precise estimation of concentration difficult for some species (e.g., Rumbo et al., 1995). However, EAG recordings can be used successfully to demonstrate which factors affect the plume structures (Suckling and Angerilli, 1996; Karg and Suckling, 1997; Suckling and Karg, 1997a,b; Karg and Sauer, 1995, 1997). For example, EAG recordings have indicated that polyethylene pheromone dispensers generate filaments or fluctuations in the pheromone, which show up as pulses or spikes in a recording. Removal of the dispensers causes the immediate cessation of pulses (Karg and Suckling, 1997). However, a certain level of behavioral disruption continues for at least one night after dispenser removal, depending on the dose of pheromone initially applied (Suckling et al., 1996; Suckling and Karg, 1997b). Hence, foliage is known to play an important part in maintaining the atmospheric pheromone concentration, by reducing windspeed and thereby limiting the losses of pheromone from the orchard (Raupach, 1988). The amount of pheromone released from foliage is small and rather hard to detect (Suckling et al., 1996), although the uptake by leaves varies with the square of distance from dispensers (Suckling et al., 1996). It is, therefore, probable that leaves within the vicinity of dispensers also release pheromone in accordance with the square of distance from the original source.

In this paper, we compare insect trapping, wing-fanning behavior, and results from electroantennogram recordings with predictions from a Lagrangian model that we have previously validated by using sampling of airborne pheromone concentrations and chemical analyses. These behavioral and physiological assays were conducted over different time scales (7 days, 2 hr at dusk, 10 min). The trapping experiments were carried out to determine threshold pheromone concentrations for flight behavior, while the wing-fanning bioassay indicates the ability of male moths to respond to pheromone stimuli in a pheromone-treated environment. The field EAG provides insights into the temporal distribution and return periods of pheromone bursts in orchard treatments with different numbers of dispensers and with different release rates. The predicted airborne pheromone concentrations under the different environmental conditions have been compared with the contemporaneous biological experiments. With the help of the predictive model, we hope to more precisely determine the environmental and operational conditions under which mating disruption is most

and least effective. Knowledge of these conditions will assist us to find ways of improving mating disruption methods.

METHODS AND MATERIALS

Field Sites. Experiments were carried out at Lincoln Springs apple orchard (Canterbury, New Zealand) in February and March 1996 during a peak of flight activity of male *E. postvittana*. The five treatments (0, 10, 100, 1000, and 2000 dispensers/ha) were established in orchard blocks that were approximately 1 ha in size and separated by shelter belts of approximately 8 m height. The apple trees were ca. 4 m tall and planted at a spacing of 2.2 m in rows that were 4.5 m apart. The dispensers were evenly distributed in each block, except for the block in which 2000 dispensers had been applied. Here, two dispensers were used at each release point (1000/ha). Dispensers were applied along the top wire in each treated block. Measurements of 50 dispensers in each block were used to determine the dispenser heights (1.74 ± 0.08 m in the block treated with 2000/ha, 1.88 ± 0.10 m in 1000/ha, 1.80 ± 0.07 m in 100/ha, 1.72 ± 0.08 m in 10/ha). Traps were checked weekly (February 2–April 19, 1996), and the number of male *E. postvittana* caught was recorded.

Meteorological Data. Air temperature, windspeed, and wind direction in the center of the 2000-dispenser plots were recorded at 1 tree height (4.0 m) on a data logger (model CR10, Campbell Scientific, Logan, Utah) by using a temperature probe (model 107, Campbell Scientific), cup anemometer (A101M, Vector Instruments), and a wind vane (W200P, Vector Instruments). In addition, a mobile data logger station was used to measure temperature and windspeed at three heights (either 0.5, 1, and 2 tree heights, or 0.25, 0.5, and 1 tree height) each time the electroantennogram recordings were made.

Tree Canopy. The approximate shape of the trees was recorded from measurements of tree height and canopy base height. The canopy radius (R_c) was recorded from the trunk to the tip of the branch at 1.0, 1.5, 2.0, 2.5, and 3.0 m heights. The leaf area index was assumed equal to 2.7, which is typical of well-managed New Zealand apple orchards (J. Palmer, personal communication).

Pheromone Dispensers. Pheromone dispensers were obtained from Shin-Etsu Chemical Co., Tokyo, and contained approximately 55 mg (*E*)-11-tetradecen-1-yl acetate (*E*11–14 : OAc), 2.5 mg (*E*)-9,(*E*)-11-tetradecadien-1-yl acetate (*E*9,*E*11–14 : OAc), 19.7 mg of the behavioral inhibitor (Rumbo et al., 1993) (Z)-11-tetradecen-1-yl acetate (Z11–14 : OAc), as well as stabilizers. The liquid length of aged dispensers applied in the treatments was measured initially for prediction of instantaneous pheromone release rates (Bradley et al., 1995). The predicted pheromone loss (decrease in liquid length) was compared to the actual loss measured on a weekly basis.

Traps. Five delta traps with sticky bases (Suckling and Shaw, 1990) were established near the middle of each treatment at two different heights (0.5 and 1 tree height) with one central trap surrounded by four other traps at a distance of 4.5 m. The traps were baited with red rubber caps (Arthur H. Thomas) loaded with 1000 μg *E*11–14 : OAc and *E*9,*E*11–14 : OAc in the ratio 100 : 5 (Bellas et al., 1983). This lure was used because our previous work had shown trap catch with this 10-fold higher loading can be significantly improved compared to standard traps in both pheromone-treated and untreated plots (Suckling and Karg 1996). The aim was to compare the disruption of trapping with predicted pheromone concentrations across a range of meteorological conditions, at two heights in the orchard.

Wing Fanning. The aim was to establish the pheromone concentration required to prevent insects from wing fanning by calculating the mean concentrations in orchard air during the time the bioassays were carried out. Wing fanning has been demonstrated to be an earlier step in the hierarchical sequence of behavior leading to mate location (Baker and Cardé, 1979; Elkington and Cardé, 1984). This behavior was chosen because it is easier to elicit (Bartell and Shorey, 1969) and to score (for presence or absence) by using moths in cages in the field at dusk.

One- to three-day-old *E. postvittana* were individually transferred from plastic bags into small cages. The cages were made from plastic Petri dishes with the lid and base of the dishes separated by a cylindrical metal mesh spacer of approximately 5 cm height. These cages allowed free air passage and permitted insect behavior to be readily observed at dusk through the clear plastic ends. The cages were hung along the upper wire (1.74–1.88 m) in four treatments ($N = 36$ moths at one time per treatment). Only four treatments (0, 10, 100, and 1000/ha) were included in this assay to permit all treatments to be performed during a single dusk period by two people. The insects were exposed to the atmosphere for 4–5 hr on the first night before the bioassay commenced. At dusk (between 20 : 30 and 23 : 00 hr), the caged insects were stimulated with pheromone puffs from a device containing a rubber septum loaded with 100 μg of pheromone. The insects received between 5 and 10 pheromone pulses (each approximately 2 ml air blown across the rubber septum). Their behavior was monitored, and wing fanning was recorded as present or absent. Each insect was given a maximum of 45 sec to respond, because preliminary experiments showed that insects rarely responded by commencing wing fanning after this time. The insects were left in the field and used in the bioassay for up to three consecutive nights. Measurements were taken on 11 nights (over four weeks). The order of treatments was randomized each night. The experiment was terminated when the dusk temperatures in the field dropped below 11°C, which is the threshold required for flight behavior (Danthanarayana, 1975). The behavior was monitored by using a red light for illumination, when required.

Electrophysiology. EAG signals were recorded in the field (Sauer et al., 1992; Karg et al., 1994) for 12 min. EAGs started with three calibration pulses

(0.5 sec) during the first minute, followed by a continuous time series measurement (10 min), and terminated with three more calibration pulses. The pulses were known air volumes (15 ml) delivered by a small pump over a rubber septum loaded with 100 μg of pheromone (for details see Karg et al., 1994). After the initial calibration period (1 min), the charcoal filter was removed and orchard air containing pheromone was sampled for 10 min (N = 8–10 replicates). The time series were processed and analyzed as described in Suckling and Angerilli (1996) and Karg and Suckling (1997). Each series was normalized to the mean of the associated calibration pulses. The EAG signals were partly corrected for drift by using a moving average of 10 sec. A long time series (10 min duration) was used to minimize the error associated with estimating the pheromone pulse return period at low dispenser densities (Suckling and Angerilli, 1996).

EAG recordings were made in all five treatments (control, 10, 100, 1000, and 2000) in the center of the experimental plots (at 1.5 m height). Pulse frequencies of the normalized EAG recordings were assessed by using the threshold of depolarization greater than -0.1 [a signal to noise ratio of 2 : 1 determined from the recordings taken in untreated control blocks (Suckling and Angerilli, 1996)]. Only pulses with EAG amplitudes exceeding this threshold were counted as a pheromone response. The frequency and return period of pulses arriving at the antennae in blocks with different numbers of dispensers was assessed. EAGs were recorded together with meteorological data. Our hypothesis was that the number of pheromone pulses increases with increasing number of dispensers per hectare.

Statistical Analysis and Modeling. Trap catch data were transformed [log (count + 1)], and the treatment and height effects were analyzed by using a two-way analysis of variance. The percentage of insects exhibiting wing fanning was angular transformed and compared between treatments. The percentage disruption was calculated for traps and bioassays as 100- [(treatment/control) $\times$ 100].

The frequency of EAG pulses (above the signal-to-noise threshold) was determined from normalized traces (Suckling and Angerilli, 1996), and pulses were placed into three classes according to (unitless) amplitude (0.1–0.19, 0.2–0.29, 0.3–0.39). Return periods were calculated as the reciprocal of frequency (time between pulses).

Meteorological and canopy measurements were used to predict atmospheric pheromone concentrations during intervals of trapping, bioassays, and EAG recordings. Estimates were based on the major component *E*11–14 : Ac only (the minor component, *E*9,*E*11–14 : Ac at 5% of the blend was assumed to behave the same over this period).

RESULTS

Pheromone Release Rate. Measurements of the liquid length of pheromone in dispensers (Figure 1, upper graph) estimate the rate of pheromone release

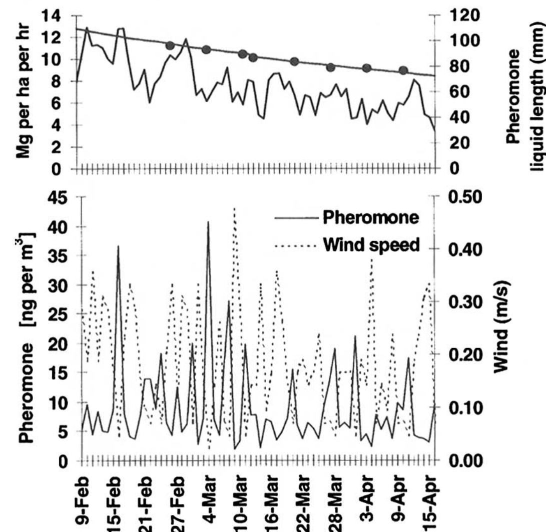

FIG. 1. Temporal change in pheromone liquid length and pheromone release rate over time (upper) and the modeled atmospheric concentration at dusk (lower) (from Bradley et al., 1995), as related to the corresponding wind speed at tree top level.

from the dispensers according to Bradley et al. (1995). Gradual changes in liquid length between each measured period were used with the air temperature to calculate the pheromone release rate on an hourly basis (Figure 1, upper graph). These predictions were then combined with other parameters such as windspeed (Suckling et al., 1999) to predict atmospheric pheromone concentrations at dusk (Figure 1, lower graph). The model showed windspeed to have a major impact on atmospheric concentrations, which we estimate to have varied between 2 and 40 ng/m^3 at dusk during the experimental period.

The predicted vertical profile of pheromone during the study showed the highest pheromone concentrations in the plane of the dispensers (1.88 m, Figure 2) and slightly lower concentrations nearer the top of the tree canopy.

Trap Catch. A total of 506 male moths were caught during the 11-week trapping period. There was a strong relationship between catch and dispensers density (Table 1). As few as 10 dispensers/ha led to 70% reduction in trap catch compared to the controls. Catch was almost zero with 1000 or 2000/ha, despite the use of high-strength (1 mg) lures.

No statistically significant effect was observed with trap height (Table 2), although an interaction between height and treatment was present. This was due to the higher catches in the lower traps in the plot with 10 dispensers/ha and in the upper traps in the plot with the 100 dispensers/ha. The difference in predicted

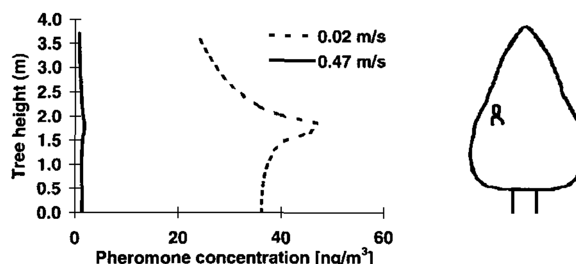

FIG. 2. Vertical distribution of modeled atmospheric pheromone concentrations at dusk in the block treated with 1000 dispensers/ha, showing high (March 4, 0.02 m/sec) and low (March 9, 0.47 m/sec) concentration profiles over the study period as a function of wind speed measured at 1.8 m. Cartoon shows apple tree with polyethylene tubing pheromone dispenser.

mean concentration at the two trap heights (1.88 and 3.76 m) was relatively small, often under twofold (e.g., Figure 2), which could account for the lack of effect of trap height on catch.

There was a reasonable correlation between percentage disruption of traps and the pheromone concentration as predicted by the model using the average meteorological conditions during dusk each week (Figure 3). The degree of correlation of predicted atmospheric concentration was similar for traps at 1.5 m (r^2 = 0.46) and 3.0 m heights (r^2 = 0.50). The percentage of disruption at 3.0 m (60–70%) coincided with predicted pheromone concentrations of ca. 1 ng/m^3 (Figure 3) and occurred during peak catches in the control traps. No catches were made when the predicted pheromone concentrations exceeded 16 ng/m^3.

TABLE 1. TRAP CATCH AND DISRUPTION OF CATCH OVER 70 DAYS AS A FUNCTION OF DISPENSER DENSITY AND DISPENSER HEIGHT[a]

Treatment (dispensers/hr)	Moths caught (mean ± SD)		Disruption (%)	
	1.5 m	3.0 m	1.5 m	3.0 m
0	37.8 ± 16.3a	35.0 ± 19.8a		
10	12.4 ± 3.5b	9.0 ± 4.5b	67	74
100	1.6 ± 1.1c	4.8 ± 2.9c	96	86
1000	0.4 ± 0.5d	0.0 ± 0.0d	99	100
2000	0.2 ± 0.4d	0.0 ± 0.0d	99	100

[a]Traps were baited with 1000-μg lures (10-fold higher than standard lures). Column entries with different letters were significantly different by ANOVA ($P < 0.05$). Fisher's LSD was used to indicate individual significant differences.

TABLE 2. ANALYSIS OF VARIANCE OF TRAP CATCH AS A FUNCTION OF PHEROMONE TREATMENT AND TRAP HEIGHT

Source	*df*	Sum squares	Mean square	*F* ratio	*P*
Treatment	4	16.75	4.188	142.63	0.001
Height	1	0.0	0.001	0.04	0.84
Interaction	4	0.42	0.104	3.54	0.01
Error	40	1.17	0.029		
Total(Adj)	49	18.34			

The tightness of the correlation is likely to be adversely affected by variations in atmospheric concentration each night over the week-long sampling interval (Figure 1). This may be true especially at the lower rates of point sources where atmospheric conditions could cause major differences in pheromone concentration due to the less even spatial distribution.

Wing-Fanning Bioassay. Wing fanning was observed in all treatments in response to the stimulus of pheromone from a 100-μg rubber septum. A dose–response was evident among treatment blocks (Table 3), but wing fanning was not completely disrupted, even at 1000 dispensers/ha. Disruption of wing fanning was positively correlated with the log of predicted atmospheric pheromone concentration (Figure 4), but considerable scatter was evident. Weather conditions during the 11 nights did not lead to predicted pheromone concentrations exceeding 10 ng/m^3, except on one occasion without wind (0.1 m/sec). On this occasion, no wing fanning was observed, since pheromone concentrations were predicted to rise above 16 ng/m^3 and thereby shut down wing fanning.

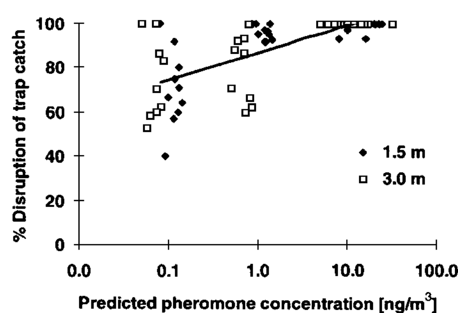

FIG. 3. Disruption of trap catch as a function of predicted weekly atmospheric concentration of pheromone ($y = 5.4\text{Ln}(x) + 87$, $R^2 = 0.54$, $P < 0.001$).

TABLE 3. WING FANNING OF MALE *E. postvittana* MOTHS IN ORCHARD AS A FUNCTION OF PHEROMONE DISPENSER DENSITY[a]

Treatment (dispensers/ha)	Observations (N)	Wing fanning (%)	Disruption (%)
0	409	88.4 a	
10	381	76.6 ab	13
100	363	61.3 b	31
1000	285	24.4 c	72

[a]Column entires separated by different letters were significantly different (ANOVA on angular transformed data, $F_{3,32} = 20.99$, $P < 0.0001$).

Electrophysiology. EAG traces of 10 min duration were characterized by a significant increase in pulse frequency with number of point sources per ha ($F_{3,30} = 4.0$; $P < 0.05$), with pulses of different amplitude (concentration) (Figure 5). Conversely, treatments showed decreasing pulse return periods for pheromone pulses of all concentrations (Figure 6). There was no significant difference in number of pulses with 1000 or 2000 dispensers/ha ($P < 0.05$) (possibly because the number of point sources was the same). The number of pheromone pulses detected in a 10-min period was weakly correlated with the predicted pheromone concentration (Figure 7). This simply reflects the increase in pulses with the increased number of point sources (Figure 6) under different weather conditions.

DISCUSSION

Our results show a reduction in trap catch with increasing atmospheric pheromone concentrations. Small amounts of pheromone (<1 ng/m^3) were able to reduce the trap catch (although there was considerable scatter in the relationship over the weekly interval). Concentrations averaging ca. 10 ng/m^3 were

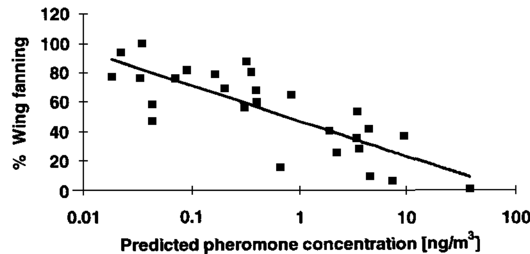

FIG. 4. Disruption of wing fanning as a function of predicted atmospheric concentration of pheromone ($y = -2.18x + 61.8$, $R^2 = 0.33$, $P < 0.001$).

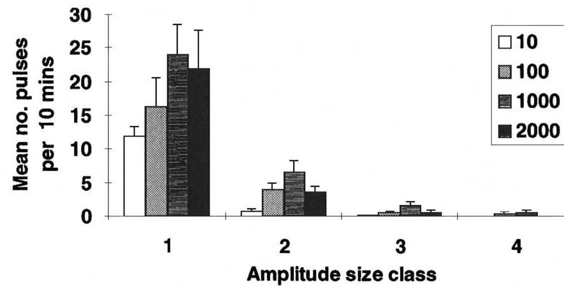

FIG. 5. Number of pheromone pulses of four size classes detected by electroantennogram in treated apple orchards, with four rates of pheromone dispensers per hectare. EAG pulse size classes are normalized to a rubber septum calibration of 100 μg of pheromone (1: 0.1–0.19; 2: 0.2–0.29; 3: 0.3–0.39; 4: >0.40).

able to prevent trap catch altogether. Wing fanning, an early step in the hierarchical behavioral sequence that leads to mate location (Elkington and Cardé, 1984), was also reduced with increasing atmospheric pheromone concentrations, although the threshold for preventing the response was closer to 70 ng/m^3. These values bracket the estimate of 20 ng/m^3 required for disruption of male oriental fruit moth behavior (Sanders and Lucuik, 1996), which was calculated as the time-averaged concentration in a uniform pheromone cloud generated from rubber septum in a wind tunnel, but is one of the few similar estimates of the pheromone concentration required for disruption.

Our results suggest that higher atmospheric concentrations may be required to arrest the wing-fanning response compared with the prevention of flight to traps. Therefore, disruption of trapping in this species may occur later in the behavioral sequence than was previously thought. Higher concentrations of pheromone are required to elicit subsequent elements in the behavioral sequence

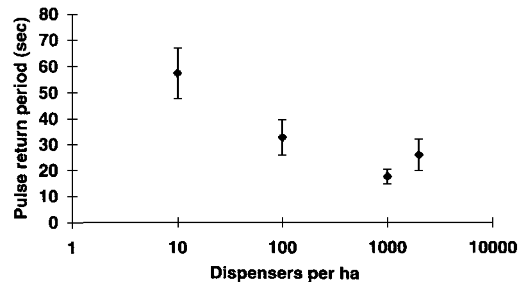

FIG. 6. Pheromone pulse return period (seconds between pulse arrival at the detector) for all classes of pheromone pulse amplitude, in relation to number of dispensers per ha.

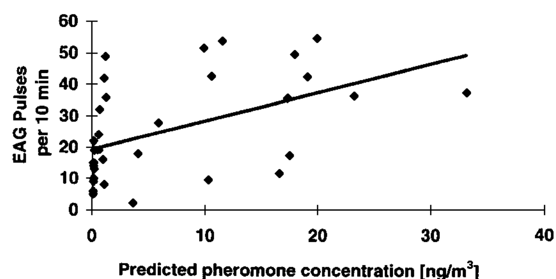

FIG. 7. Relationship between pheromone pulse frequency (all classes) and predicted pheromone concentration in orchard air ($y = 0.92x + 19.37$, $R^2 = 0.26$, $P < 0.001$).

(Bartell and Shorey, 1969). However, the proportion of moths that complete a full behavioral sequence may be a function of the previous exposure to pheromone, as well as the stimulus concentration (Sanders and Lucuik, 1996), and our results involved exposure of the insects to pheromone fluctuations under natural conditions. It is possible that the apparent difference in threshold values of the two behavioral characteristics was influenced by the proximity of the stimulus to males. In the wing fanning assay, a 100-μg loading was used, compared to traps that used a 1000-μg loading. However, if the pheromone concentration is maintained within air filaments over substantial distances within the orchard air, as was found in open air (Murlis and Jones, 1981; Murlis, 1986, 1996), then this loading factor may be less important. Another difference between the two experiments was the integration interval, although it is unclear how this could affect the accuracy (as opposed to precision) of our observations.

Our results show that the moths are able to retain their ability to respond to the pheromone in the field, provided that it is presented at a sufficiently high dose relative to that of the background. Here, the stimulus dose used to promote wing fanning (100 μg) was considerably more than the pulses typically found in orchard air detected by the electroantennogram. Rather, the equivalent doses to these stimuli would be similar to the normalizing pulses used to calibrate the EAG traces (i.e., the normalized value of 1.0), while the maximum EAG pulse detected in orchard air was 0.4.

Our predictions of pheromone concentrations at 1.85 m above ground at dusk varied between 4 and 90 ng/m^3 for plots treated with 1000 dispensers/ha. This variation was determined mainly by windspeed. We have previously found that vertical windspeed from turbulent gusts is the factor most correlated with antennogram descriptions of concentration in real time (Suckling and Karg, 1997a). Our Lagrangian model was previously found to be of sufficient accuracy (i.e., within a factor of two of measured concentrations) in blocks treated with 1000 dispensers/ha (Suckling et al., 1999). While factors such as air temperature,

dispenser release rate, and foliage density affect the atmospheric concentrations of pheromone, other diurnal changes, such as windspeed, appear likely to be more important in determining the efficacy of disruption.

Windspeed in orchards typically increases with height above ground, with higher speeds producing greater vertical mixing within the canopy airspace (Suckling et al., 1999). The extent of turbulent mixing was probably sufficient to prevent any significant difference between trap catch over the heights investigated here. In a study in a small (0.5 ha) isolated hill-top orchard block, Suckling and Shaw (1992) reported that mating rarely occurred in blocks treated with 1000 dispensers/ha, and then only at 3.0 m (not 1.5 m). Lawson et al. (1996) also failed to detect any significant difference in mating disruption with height.

The EAG recordings showed decreasing pulse return periods (i.e., more pheromone pulses per unit time) with increased point source density, plotted on a log scale. These results corroborate earlier findings by Suckling and Angerilli (1996). The relatively poor correlation between pulse return period and atmospheric concentration could be due to the confounding effect of wind, which increases pulse frequency (Karg and Suckling, 1997), but decreases atmospheric concentration (Suckling et al., 1999).

Karg and Suckling (1997) showed that the complete removal of dispensers from a treated orchard resulted in a gradual drop in the mean EAG values (background concentration) over 1 hr. In addition, the pheromone filaments detected by EAG disappeared upon removal of the dispensers, although the pheromone being released from the foliage sources was still enough to cause disruption of trap catch for one night. We conclude that the fluctuations in the EAG signals provide a method for detection of the near-field concentration (Suckling et al., 1999) produced by the dispensers nearest to the antenna, while the contribution of the background mean concentration is due to more distant dispensers (far-field concentration).

Application of the Lagrangian model to the two lower rates of pheromone (10 and 100 dispensers/ha) violates the assumption of uniform source distribution in the canopy by having excessively large distances between dispensers, and this probably contributes to the large scatter in the correlations between both trap catch and wing fanning and concentration. However, predictions from the model did improve the correlation between pulse frequency and pheromone expressed as dispenser rate or as the predicted concentration.

The Lagrangian model offers an improvement over earlier attempts to understand the factors affecting the efficacy of mating disruption, and has proven to be a useful tool to describe experimental results for periods ranging from days (trapping), hours (wing fanning), to minutes (EAG traces). The concentrations required for disruption of different measures of efficacy (trapping or wing fanning) appear to be different, and further investigation is needed to elucidate this and the importance of average concentrations compared to plume structure to dis-

ruption in the field. We believe that parallel development of both measurement and modeling techniques is the best, if not the only way, to track our progress in developing a more precise understanding of mating disruption.

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PIPERIDINE ALKALOIDS IN NITROGEN FERTILIZED *Pinus ponderosa*

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Abstract—We fertilized individual, pole-size ponderosa pine trees at two low-quality sites and pine saplings at a relatively high-quality site, with ammonium nitrate. Six to 12 months later, we measured total %N and 2,6-disubstituted piperidine alkaloids in the foliage. The N additions raised foliar %N above deficiency levels (i.e., from 1.0–1.1% to 1.4–1.6%) at the low-quality sites, but did not elevate foliar %N in saplings at the higher quality site, where it was already (1.9%) well above critical levels. In control trees with foliar N below a threshold of 1.1%, we detected no more than trace levels of alkaloids, indicating that alkaloid production is highly constrained by N deficiency. The N additions increased mean concentrations of the predominant alkaloid, pinidine, at all three sites. Mean total alkaloid concentrations for fertilized trees at the two low-quality sites were 12 and 155 $\mu g/g$ dry wt higher than controls (relative increases of 12× and 4.5×, respectively). For saplings at the high-quality site, the mean total increased by 584 $\mu g/g$ dry wt (1.6×) with the N additions. Allocation of foliar N to alkaloids was highest in fertilized saplings (0.81%) compared to control saplings (0.53%). These findings demonstrate that foliar alkaloid concentrations can be increased by nitrogen fertilization of forest trees growing on both low- and high-quality sites. Fertilizing for the purpose of inhibiting potential herbivores may be more successful at higher quality sites where alkaloid levels are enhanced relative to food quality (foliar %N).

Key Words—2,6-Disubstituted piperidine alkaloids, *Pinus ponderosa*, ponderosa pine, fertilization, nitrogen availability, foliar nitrogen, Pinaceae.

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INTRODUCTION

Ponderosa pines, *Pinus ponderosa* Dougl. ex Laws., have *cis*-2,6-disubstituted piperidine alkaloids in their foliage (Tallent et al., 1955; Stermitz et al., 1994), which may contribute to chemical defense against herbivores or pathogens (Schneider et al., 1991; Tawara et al., 1993; Stermitz et al., 1994; Kamm et al., 1998). Concentrations of the Pinaceae alkaloids vary qualitatively and quantitatively within and among sites (Tawara, 1994; Todd, 1994; Tawara et al., 1995; Todd et al., 1995; Gerson and Kelsey, 1998), but the extent to which these alkaloid profiles are environmentally controlled is poorly understood. Observational field studies have shown positive correlations between nutrient availability and alkaloid levels in trees (Gerson and Kelsey, 1998; Hoft et al., 1998), but a causal relationship could only be suggested by these studies. Controlled experiments have determined that nutrients, light, water, and herbivory can interact to influence foliar alkaloid levels in a variety of plants (e.g., Johnson et al., 1987; Krejsa et al., 1987; Baldwin and Ohnmeiss, 1994; Hoft et al., 1996; Ralphs et al., 1998), but such relationships have not been established for conifers.

Of the environmental variables known to influence alkaloids, nutrients are most readily controlled in the silviculture of pines. Light levels can be altered by thinning, but concurrent effects on water and nutrient levels would confound the light treatments. Fertilizing pine forests has been shown to increase foliar concentrations of other secondary metabolites (Bjorkman et al., 1991; McCullough and Kulman, 1991), but effects on herbivores have been mixed (e.g., Smirnoff and Bernier, 1973; Bjorkman et al., 1991; McCullough and Kulman, 1991; Wickman et al., 1996). Ponderosa pine is widely distributed in the western United States, often growing in harsh environments where soil fertility, droughty conditions, and temperature extremes contribute to nutrient limitations. Ponderosa pine forests also are subject to large-scale outbreaks of defoliators (Furniss and Carolin, 1977) that can seriously impact forest values (McMillin and Wagner, 1989). Thus, we were interested in ascertaining whether nitrogen additions could be used to increase foliar alkaloid concentrations under field conditions.

Our previous work with ponderosa pine in central Oregon established that foliar alkaloid levels can vary dramatically among sites (Gerson and Kelsey, 1998). Alkaloid concentrations were strongly correlated with stand density, and foliar nitrogen explained some of the variability, but genetic variation also was thought to contribute to the striking differences in alkaloid concentrations among the three sites. Trees from one site (site A) had virtually no alkaloids. This site carried a densely packed stand of small-diameter trees with 28 m^2/ha basal area on infertile pumice soil. The stand was planted in the 1940s, probably from an offsite seed source, and, therefore, most likely to be genetically distinct from the two naturally regenerated stands. We returned to site A for the current study because we knew these trees were alkaloid deficient and would provide a good

test for our fertilizer treatments. For comparison, we also fertilized individual trees at site C from the previous study, which had alkaloids in every tree sampled. To test the effect of nitrogen additions on ponderosa pine with high alkaloid levels (Gerson and Kelsey, 1999), we randomly fertilized individual saplings in a Willamette Valley, Oregon plantation (hereafter, site D).

METHODS AND MATERIALS

Site Descriptions. Three sites were chosen to represent a range of inherent nitrogen availability and piperidine alkaloid levels in ponderosa pine. The lowest quality site (A) and the intermediate site (C) are located in the Deschutes National Forest (N.F.) in central Oregon and are the same sites previously described in detail (Gerson and Kelsey, 1998). The same site labels (i.e., A and C) are retained here for continuity, although different sample trees were selected. There is no site B designated in this paper. Annual precipitation and site productivity are approximately 38 cm and 3.0 m^3 biomass/ha/yr, respectively, for site A; and 38–43 cm and 3.75 m^3 biomass/ha/yr, respectively, for site C (Bend-Ft. Rock Ranger District, Deschutes N.F., records on file). Foliar %N, measured in August 1996 (Gerson and Kelsey, 1998), averaged 1.0% at site A and 1.3% at site C in current-year foliage; in previous-year foliage, foliar %N averaged 1.2% at site A and 1.5% at site C. Available N in soil was 4.7 $\mu g/g$ at site A and 18.0 $\mu g/g$ at site C. Mean total alkaloid concentrations measured in previous-year foliage in April 1996 were 0 $\mu g/g$ at site A and 33 $\mu g/g$ at site C. Trees at site A were planted 50–60 years ago; trees at site C were naturally regenerated and approximately the same age.

The highest quality site (D) is located roughly 150 km west of sites A and C at Peavy Arboretum (4945 km N, 482 km E, UTM zone 10; 140 m elevation) in Oregon State University's McDonald Research Forest. Trees at site D were 3- to 4-year-old saplings from a Willamette Valley seed source (zone 262-0.5). Annual precipitation and site productivity are approximately 102 cm and 11.3 m^3 biomass/ha/yr, respectively. We had no prior knowledge of foliar %N or available N in soil at this location, but we knew the trees were likely to have high alkaloid concentrations because a few had been sampled for a methodology study (Gerson and Kelsey, 1999).

Fertilizer Application. At sites A and C, eight pairs of trees were identified based on proximity and attributes (similar diameter, height, crown). One tree from each pair was randomly selected to receive the nitrogen treatment, the other was a control. On May 1, 1997, a broadcast spreader was used to apply nitrogen, phosphorus, and sulfur (or just phosphorus and sulfur for controls) in a 100-m^2 circular plot around the sample trees. The application rates were 224 kg N/ha, 112 kg P/ha, and 34 kg S/ha, as ammonium nitrate (NH_4NO_3), triple super phos-

phate [$Ca(H_2PO_4)_2$], and gypsum ($CaSO_4{\cdot}2H_2O$), respectively (Cochran, 1973). Phosphorus and sulfur were applied, in addition to nitrogen, at sites A and C because of deficiencies in central Oregon pumice soils (Cochran, 1978; Will and Youngberg, 1978). Existing vegetation (bitterbrush, grasses) within the plots was not removed.

At site D, 32 saplings in good condition were identified, and competing vegetation (grasses, bracken fern, poison oak) was controlled with glyphosate and manual removal prior to fertilizer application. On May 12, 1997, sixteen of these saplings were randomly selected to receive the fertilizer treatment: ammonium nitrate at 224 kg N/ha over a 1-m^2 circular plot. Control trees were not provided with any nutrients. Two days later, 4 liters of water was sprinkled over each circular plot to dissolve any remaining fertilizer.

Foliar Sampling and Extraction. At site D, foliage from the current growing season was sampled six months after fertilization (October 27, 1997). Ten fascicles of needles were taken from lateral stems in the top whorl and stored intact in open paper bags for one to two weeks (Gerson and Kelsey, 1999). Ponderosa pine typically produces three needles per fascicle; from each fascicle we allocated two needles for alkaloid extractions and the third to nitrogen analysis. Samples were extracted as described by Gerson and Kelsey (1998), except the needles were air-dried rather than frozen, and a 10-ml aliquot of the supernatant was measured following centrifugation to improve accuracy.

At sites A and C, foliage from the 1997 growing season was sampled 12 months after fertilization (April 21, 1998). In late August 1997, nearly four months after application, some pelletized fertilizer (~5–10%) was still visible on the soil at sites A and C. These sites had received little rainfall since the fertilizer was applied; most annual precipitation in this region occurs as snow. Consequently, we postponed sampling at sites A and C until April 1998 to allow additional time for snowmelt and microbial processes to make the N available to the trees, but not so much time that the pulse of N could be diluted into the 1998 foliar growth. Two branch tips from the north aspect and two from the south were clipped from the middle third of the live crown with a pole pruner and stored separately (by aspect) in paper bags. The following day, 10–15 fascicles were taken from each branch, then dried for three days at 70°C. Dried needles were ground to pass a 20-mesh screen, a portion was allocated to N analysis, and the remainder was stored in zip-lock bags at 2–5°C until extraction. Samples from north and south aspects were treated as duplicates for the alkaloid analysis. A slightly modified methodology (Gerson and Kelsey, 1999), developed after the analysis of site D samples, was followed for site A and C samples. The use of chloroform and extra rinsing in the extraction procedure was found to improve alkaloid yields, whereas differences in the sample drying procedures were found to be inconsequential.

Nitrogen Analysis. Composite samples were prepared for each sample tree

at sites A and C by combining equal weights of ground foliage from north and south aspects. Dried needles remaining from site D samples also were ground (20 mesh). Samples were analyzed for total percentage of nitrogen following micro-Kjeldahl digestion by the Plant & Soil Analytical Lab, Department of Forest Science, OSU, by using an Alpkem-RFA 300 rapid-flow colorimetric analyzer.

Alkaloid Analysis. All known alkaloids in the foliar extracts were quantified by GC-MS as previously described (Gerson and Kelsey, 1998). These compounds were identified according to published mass spectra (Hart et al., 1967; Tawara et al., 1993) as pinidine [2-methyl-6-(2-propenyl)piperidine], pinidinol [2-methyl-6-(2-hydroxypropyl)piperidine], 1,2-dehydropinidinol [2-methyl-6-(2-hydroxypropyl)-1,2-piperideine], pinidinone [2-methyl-6-(2-oxopropyl)piperidine], 1,2-dehydropinidinone [2-methyl-6-(2-oxopropyl)-1,2-piperideine], and euphococcinine (1-methyl-9-nor-3-granatanone).

Statistical Analysis. Data from sites A and C were analyzed separately from site D because of differences in tree age, sampling, and alkaloid extraction procedures. Fertilizer effects on total N and alkaloid concentrations in foliage were tested using paired, one-tailed t tests for sites A and C, and unpaired, one-tailed t tests for site D (SAS Institute, Inc., 1989).

RESULTS AND DISCUSSION

Foliar Nitrogen. As expected from our previous work (Gerson and Kelsey, 1998), total foliar N (% dry weight basis) was lowest in controls at site A and slightly higher in site C controls (Table 1), confirming that trees at site A had the least favorable nutrient status prior to fertilization. Nitrogen deficiencies in conifers may be identified by foliar %N below a critical level required for growth. Compared to other conifers, the critical level of foliar N is low for ponderosa pine: approximately 0.9% (Oliver and Ryker, 1990) to 1.1% (Powers et al., 1988) for pole-sized trees. Nitrogen concentrations in current-year foliage of all control trees at site A fell below 1.1%, indicating these trees were N deficient. Control trees at site C had borderline deficiency levels, ranging from 1.0 to 1.2%. Not surprisingly, N fertilization increased total N in foliage at sites A and C: fertilized trees had 0.3–0.9% higher foliar N at site A, and 0.1–0.6% higher foliar N at site C, compared to controls at each site. In a similar study, mature ponderosa pine with low foliar N levels responded similarly to ammonium nitrate fertilization within one year (Zabowski and Henry, 1995).

At site D, the range of foliar N in both control and fertilized saplings (1.6–2.1%; Figure 1) was entirely above the range measured in the central Oregon trees, and the fertilizer treatment did not increase total N in the current-year foliage (Table 1). In another study, field fertilization of ponderosa pine seedlings growing at a nutrient-rich site also had no effect on foliar N concentrations (in the

TABLE 1. TOTAL NITROGEN CONCENTRATION IN FOLIAGE

Site	N	Concentration (% dry wt, mean ± SE)		Prob > |t|
		Control	N-fertilized	
A	8	0.99 ± 0.07	1.55 ± 0.15	<0.001[a]
C	8	1.10 ± 0.06	1.41 ± 0.13	0.001[a]
D	16	1.89 ± 0.10	1.93 ± 0.10	0.133[b]

[a]Paired, one-tail t test.
[b]One-tail t test.

1.6–1.9% range) or on growth of the seedlings (Gleason et al., 1990). Growth responses to the fertilizer were not measured in our study, except for needle lengths of samples from site D. We found no difference in needle length between the treatments (data not shown). From the above, we conclude that foliar N in sample trees at site D was probably near optimal.

Alkaloid Response to N Additions. Pinidine, the primary end product of piperidine biosynthesis in ponderosa pine (Tawara et al., 1993), is the most ubiquitous and abundant of the *Pinus* alkaloids in foliage from the study areas (Gerson and Kelsey, 1998, 1999). Therefore, N effects on pinidine are of particular interest. The nitrogen treatments enhanced pinidine concentrations at all

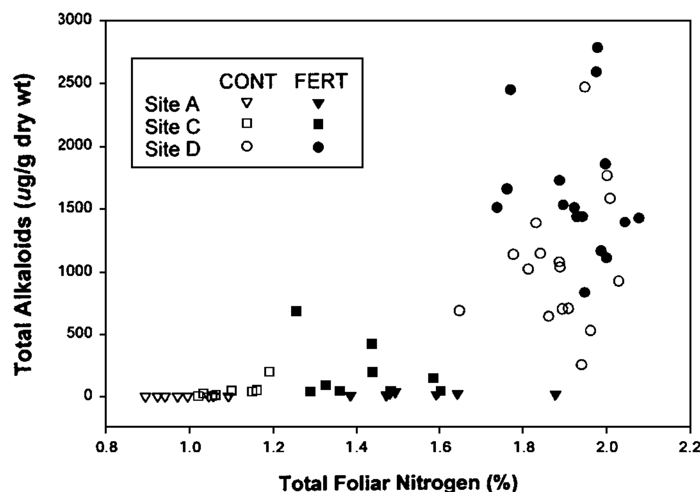

FIG. 1. Total alkaloid concentrations relative to total percentage of nitrogen in foliage. N = 8 control and 8 nitrogen fertilized trees at sites A and C; N = 16 control and 16 nitrogen fertilized saplings at site D.

TABLE 2. PIPERIDINE ALKALOIDS IN FOLIAGE AT SITES A AND C IN CENTRAL OREGON

Alkaloid	Concentration (μg/g dry wt, mean ± SE)[a]		Prob > $|t|$[b]
	Controls	N-fertilized	
Site A			
Pinidine	0 ± 0	12 ± 6	0.039
Other piperidines	0 ± 0	0 ± 0	0.999
Total	0 ± 0	12 ± 6	0.039
Site C			
Pinidine	44 ± 23	175 ± 67	0.015
1,2-Dehydropinidinol	0 ± 0	19 ± 13	0.096
Pinidinol	0 ± 0	0 ± 0	0.999
1,2-Dehydropinidinone	0 ± 0	2 ± 2	0.175
Pinidinone	0 ± 0	0 ± 0	0.999
Euphococcinine	0 ± 0	2 ± 2	0.175
Total	44 ± 23	199 ± 83	0.022

[a]$N = 8$.
[b]Paired t tests.

three sites. At site A, where practically no piperidine alkaloids were detected in our previous study, four of the eight nitrogen-fertilized trees produced 10–44 μg/g dry wt of pinidine, and none of the control trees produced any pinidine. The pinidine response was not large (Table 2), but it is meaningful in that it demonstrates the trees at this site are not absolutely (genetically) constrained from producing the *Pinus* alkaloids. A similar response (but at higher alkaloid levels) was observed at site C (Table 2). Among the eight pairs of trees, the N-fertilized tree had 9–353 μg/g dry wt more pinidine after 12 months. At site D, all 32 trees contained pinidine in concentrations ranging from 204 to 1071 μg/g in controls, and from 375 to 1235 μg/g in fertilized trees, yet the mean concentration of pinidine was significantly higher for the fertilized trees (Table 3). The high variation in alkaloid concentrations within sites was typical (Gerson and Kelsey, 1998).

Nitrogen effects varied for the other *Pinus* alkaloids, although generally the effect was stronger where alkaloids were present in higher concentrations (Tables 2 and 3). None of the *Pinus* alkaloids, other than pinidine, was detected in any of the sample trees at site A. At site C, small concentrations (15–100 μg/g dry wt) of the 1,2-piperideines and euphococcinine were found only in the two N fertilized trees with the most pinidine. Therefore, the additional N had only a marginal effect at best (all $P \geq 0.096$), on concentrations of alkaloids other than pinidine at sites A and C. At site D, most of the *Pinus* alkaloids were present in control and fertilized trees. A nitrogen effect was clear for 1,2-dehydropinidinol (Table 3), which had concentrations comparable to pinidine. Interestingly,

TABLE 3. PIPERIDINE ALKALOIDS IN FOLIAGE AT SITE D IN WILLAMETTE VALLEY

Alkaloid	Concentration (μg/g dry wt, mean ± SE)[a]		Prob > |t|[b]
	Controls	N-fertilized	
Pinidine	598 ± 59	775 ± 63	0.026
1,2-Dehydropinidinol	316 ± 84	639 ± 105	0.011
Pinidinol	42 ± 12	87 ± 12	0.056
1,2-Dehydropinidinone	58 ± 13	85 ± 17	0.105
Pinidinone	3 ± 2	8 ± 3	0.066
Euphococcinine	39 ± 5	45 ± 4	0.156
Total	1055 ± 542	1639 ± 537	0.002

[a] $N = 16$.
[b] One-tail t tests, with equal/unequal variance as appropriate.

the proportion of 1,2-dehydropinidinol in the total alkaloid sum increased by about 10%, and pinidine decreased correspondingly, in the N-fertilized saplings. Concentrations of the remaining alkaloids were below 100 μg/g dry wt, and although means were higher in all cases for the N fertilized trees, the differences were only marginally significant (all $P \geq 0.056$). The 1,2-piperideines, pinidinol, and pinidinone are thought to be biosynthetic precursors to pinidine (Leete and Juneau, 1969; Leete et al., 1975; Tawara et al., 1993, 1995), and euphococcinine is probably an alternative end product (Tawara et al., 1993), so N effects on the concentrations of intermediate compounds may have been less apparent because of their transitory nature. When cumulative sums of the alkaloids are evaluated, the N effect becomes more apparent. Total alkaloid concentrations were increased by the N additions at all three study sites (Tables 2 and 3). At site D especially, the cumulative effect was an order of magnitude stronger than effects on any individual alkaloid.

The relationship between foliar N and alkaloid levels (Figure 1) revealed that allocation of N to constitutive alkaloids is partially dependent on availability. No more than trace levels of alkaloids were observed in trees at sites A or C with <1.1% foliar N, demonstrating that alkaloid production in ponderosa pine is highly constrained when foliar N is deficient. Alkaloid synthesis in fertilized trees at site A was low in comparison to site C, relative to the increase in foliar %N at both sites, indicating that factors other than N availability also played a role. Alkaloid biosynthesis is dependent on enzyme systems that may be diminished in plants grown in nutrient-poor conditions (Johnson et al., 1989). If alkaloids accumulate early in developing leaves and are diluted with age (Ralphs et al., 1998), which appears to be the case for ponderosa pine (Tawara et al., 1995; Gerson and Kelsey, 1998), then the timing of nitrogen fertilization, relative to tree phenology, could limit the alkaloid response to N additions, even though foliar %N increases. Trees at sites A and C were phenologically similar

TABLE 4. PROPORTION OF LEAF NITROGEN ALLOCATED TO ALKALOIDS

Site	*N*	Alkaloid N/Total N (%, mean ± SE)	
		Control	N-fertilized
A	8	0.00 ± 0.00	0.01 ± 0.00
C	8	0.04 ± 0.02	0.14 ± 0.06
D	16	0.53 ± 0.09	0.81 ± 0.09

and fertilized on the same day, but N uptake could have been delayed at site A by the closed canopy or other environmental factors. Alternatively, the trees at site A may simply have been inherently low alkaloid producers (as in Krejsa et al., 1987).

At site D, where foliar N was well above deficiency levels (>1.8%), the N additions had no effect on foliar %N, and there was no correlation between foliar N and alkaloid levels (Figure 1). This apparent decoupling of alkaloid and foliar N levels could result from maximized leaf N (protein) levels and allocation of N to other tissues. The proportion of foliar N in piperidine alkaloids is so small it seems unlikely these alkaloids function as N storage compounds. Increases in alkaloid concentrations at site D, in response to what could be considered excessive N additions, differed from the response of monoterpene indole alkaloids produced by an evergreen broad-leaved tree, *Tabernaemontana pachysiphon* Stapf (Apocynaceae) (Hoft et al., 1996). By using the proportion of foliar N fixed in alkaloids as an index of N allocation, Hoft et al. (1996) noted that moderately and strongly fertilized seedlings allocated less N to alkaloids (48%) than unfertilized seedlings (0.66%). In contrast, fertilized saplings at our site D allocated 0.80% of their foliar N to piperidine alkaloids, whereas 0.53% of foliar N in control trees was incorporated in alkaloids (Table 4). Both studies used young, evergreen, woody plants; however, the involvement of different biosynthetic pathways could account for the different N allocation strategies and alkaloid responses observed for the two species. The *Pinus* alkaloids are synthesized from polyketide precursors (Leete and Juneau, 1969; Leete et al., 1975), whereas the alkaloids of *T. pachysiphon* are from amino acid/monoterpene origins.

Considering the alkaloid responses to N fertilization across the range of initial (site) conditions (Figure 1), it is apparent that foliar N is a meaningful correlate for alkaloid levels under limited circumstances (perhaps for coarse-scale, but not fine-scale comparisons) and that factors other than N availability influence alkaloid levels in ponderosa pine foliage. Mature trees at sites A and C allocated a very small percentage of their foliar N to alkaloids even when leaf N was sufficient, compared to saplings at site D (Table 4). Tree age is a potential factor, in addition to the environmental and genetic differences already discussed, that could have contributed to the variation between the central Oregon

and Willamette Valley sites. Young trees can have N allocation strategies different from older trees (Kozlowski and Pallardy, 1997). For example, protein (and procyanidin) can increase in ponderosa pine seedlings and decrease in pole-size trees in response to defoliation (Wagner, 1988). Hoft et al. (1998) found slightly lower indole alkaloid levels in older *T. pachysiphon* trees. Our data are consistent with the possibility that younger ponderosa pine allocate more foliar N to alkaloids. Piperidine alkaloids accumulate early in seedling growth (Tawara, 1994; Todd, 1994; Tawara et al., 1995; Todd et al., 1995). There have been no controlled comparison of piperidines among young and mature conifers, however. In fact, information regarding the effects of nutrient additions of N-based secondary metabolites comes primarily from greenhouse studies of young plants, and allocation of N to alkaloids in mature woody plants is relatively undocumented.

As noted in our previous study (Gerson and Kelsey, 1998), light herbivory on ponderosa pine does not appear to induce piperidine alkaloid production in existing foliage. Some herbivory was noted on the samples collected at sites A and C in central Oregon, where an outbreak of the pandora moth (*Coloradia pandora* Blake) had recently subsided. Several larvae of this pine defoliator were found in foliage samples from site C. The larval stage of *C. pandora* occurs biennially and was out of phase when we fertilized in May 1997, but larvae emerged the following September, overwintered on the foliage, and fed as temperatures allowed until June 1998. The branches sampled in April 1998 for fertilizer effects were examined for defoliation and assigned an herbivory index denoting the extent of damage. Regressions of these herbivory indices on total alkaloid concentrations at each site indicated no relationship between the variables and confirmed our previous observations. In central Oregon, *C. pandora* larvae complete their feeding stage about the same time that new needles begin elongating, and it remains possible that alkaloid levels in the new foliage could be influenced by herbivory on the older foliage.

Effects of the *Pinus* alkaloids on *C. pandora* larvae have not been tested directly, but fertilization of ponderosa pine in another central Oregon study (Wickman et al., 1996) increased foliar N levels to 2.1%, reduced *C. pandora* larval weights approximately 30%, and increased N in larval frass. These effects on the *C. pandora* larvae may be explained by fertilizer-induced increases in foliar alkaloids and their subsequent excretion [as in Kamm et al., 1998, for western spruce budworm, *Choristoneura occidentalis* (Freeman)]. Fertilizing forests for the purpose of inhibiting herbivory should be weighed against potential benefits to herbivores in terms of food quality (McClure, 1991; Mason et al., 1992). The fertilizer response at site D in our study, where high foliar N levels were not increased while foliar alkaloid levels were, is particularly interesting in this respect. Assuming that %N adequately reflects nutritional quality of the foliage, we can infer that N fertilization to inhibit herbivory of ponderosa pine would

be more effective where nitrogen sufficiency is established (e.g., at high nutrient sites or with repeated fertilizer applications). This inference depends on a quantitative sensitivity of the herbivore to the piperidines, which remains to be demonstrated. Preliminary studies of antifeedant activity by the Pinaceae alkaloids toward lepidopteran larvae (Schneider et al., 1991; Stermitz et al., 1994) have had mixed results (F. R. Stermitz, personal communication).

SUMMARY AND CONCLUSION

Nitrogen fertilization increased total alkaloid concentrations in ponderosa pine foliage at two low quality sites and also at a high quality site. Fertilization increased total N in foliage at the low-quality sites, but not at the high quality site. This study demonstrated that fertilization of ponderosa pine, and by extension, other silvicultural treatments that increase N availability (such as thinning overly dense stands), might be used to increase alkaloid concentrations in foliage. If forthcoming antifeedant research indicates that promoting alkaloid production in pine forests would be desirable, our findings suggest that fertilizing trees that already have high foliar N would likely be more successful in deterring herbivory because alkaloid levels would increase without a concomitant increase in foliar nutritional quality.

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AQUACULTURAL EFFLUENTS: DIRECTIVE SIGNALS TO THE SYSTEM DOWNSTREAM?

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Abstract—Animals continuously release biogenic substances that vary in composition with physiological state. In aquatic systems, animals can gain insight about conditions or events upstream and alter their physiology and behavior to exploit this information. Here, we review observations on aquatic animals as diverse as snails, shrimp, fish, and frog tadpoles to probe the possibility that high-density aquaculture might generate chemical messages that cause conspecific or related individuals to reduce productive processes (growth, metamorphosis, ecdysis, reproduction) or even to sicken and die (loss of immunocompetence, anaphylaxis). The potential for ecological disruption logically is maximized under conditions that uncouple the parts of the system generating and receiving such signals—as would be the case when aquacultural effluents enter natural aquatic systems.

Key Words—Pheromone, Schreckstoff, metabolite, exocrine, directive factor, semiochemical, endocrine disruption.

INTRODUCTION

The survival and growth of organisms typically is density-dependent. Most aspects of density dependence are readily explained as physiological consequences of resource insufficiency (e.g., low oxygen) or metabolite excesses (e.g., high ammonia). However, there is increasing evidence to suggest that reproduction, growth, feed efficiency, and even survival of aquatic animals are influenced by semiochemicals. Semiochemicals are substances that have information value that is separate from known physiological effects (Pasteels, 1982), that is, semiochemicals have directive effects, in the sense of Fry (1947). Implicitly, directive

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signals have adaptive value to the sender and/or the recipient (Pasteels, 1982). For instance, the odor of food conveys meaningful information to the recipient but does not offer any nutritive value in and of itself. The odor of a predator alarms and may repel prey species, but typically it does not do physical harm to the prey species. The olfactory bouquet of a particular stream attracts salmon that were hatched and developed there, to return and themselves spawn, even though the stream may have become a poor spawning venue owing to some ecological disruption (e.g., deforestation in the watershed). Chemical signals from a female pine bark beetle that has settled onto and burrowed into an appropriate tree will attract male pine bark beetles. The foregoing are examples of subsets of semiochemicals—attractants, allemones, ecomones, and pheromones, respectively. These subsets are mentioned to illustrate the diversity of chemical messages, but it also should be noted that there is considerable debate concerning the definitions of these terms. Burghardt (1970), Wilson (1970), and Pasteels (1982) discuss terminology relating to the various classes of semiochemicals.

Some semiochemicals in the aquatic environment may have information value that is concordant with other physiological effects. For instance, ammonia may signal overcrowding and thus act as a repellent. In addition, ammonia can act as a limiting or even lethal environmental factor (Fry, 1947, 1971) by interfering with oxygen uptake in gill-breathing animals. Such a compounded effect complicates the study of the role of semiochemicals in aquaculture. Semiochemical interactions have been explored for some aquacultured animals, such as shrimp, crayfish, trout, minnows, catfish, and tilapia. These cases are discussed below, along with potentially relevant examples involving several other aquatic animals. Additionally, the literature contains many other cases of semiochemical phenomena that may assist in understanding the biology of aquacultured species (Table 1).

SEMIOCHEMICALS AND AQUATIC SPECIES

Snails. Nassarius spp. exhibit a strong escape response to the homogenized juices of conspecifics and a lesser escape response to juices of nonconspecific congenerics. Juices of most noncongeneric mollusks instead induce a feeding response (Atema and Stenzler, 1977; Stenzler and Atema, 1977).

Alpheid Shrimp. The endogenous hormone 20-hydroxyecdysone promotes molting in several crustaceans. When applied exogenously as a 0.05-ppm solution, it can shorten the molt cycle of alpheid shrimp (Mellon and Greer, 1987).

Penaeid Shrimp. In a simple Y-maze experiment, penaeid shrimp larvae in springtime preferred water from an estuarine source over synthetic seawater (Benfield and Aldrich, 1992). However, estuarine water lost its attractiveness during the winter, suggesting that the source of the attractant was bio-

TABLE 1. SEMIOCHEMICALS IN AQUATIC SYSTEMS

Function	Organism(s)	Agent(s)	Action/Response	Citation
Recognition of Conspecifics	Gregarious polychaetes	DOPA moietes in cement (dihydroxyphenylalanine)	Induces settlment and metamorphosis in conspecific larvae	Jensen and Morse (1990)
	Gammarus palustris amphipod	Water conditioned by male, female conspecific, or congeneric	Increased probability of choosing water conditioned by conspecifics and opposite sex	Borowsky (1985)
	Orconectes virillis crayfish	Water conditioned by nonself conspecifics	Aggressive posture upon exposure to nonself water	Hazlett (1985)
	Oncorhynchus kisutch Coho salmon	Water conditioned by conspecifics	Prefers water conditioned by conspecifics	Quinn et al. (1983)
	Ictalurus natalis and *Ictalurus nebulosus* yellow and brown bullhead	Water conditioned by conspecifics, possibly mucus and urine	Multiple actions based on odor (non-learned spontaneous aggression), experience (learned submission) and environment (suppression of aggression)	Todd (1971)
Feeding	*Palaemonetes pugio* grass shrimp	Purines, purine nucleotides, homarine, lactic acid, trimethylamine oxide	Feeding	Carr et al. (1984)
	Panulirus argus spiny lobster	AMP, ATP, L-glutamate, glycine, taurine	Cells differentially activated by each compound	Carr (1992)
Habitat selection	*Penaeus aztecus* and *Penaeus setiferus* brown and white shrimp	Biogenic source in estuarine water	Prefers water of estuarine origin	Benfield and Aldrich (1992)

TABLE 1. CONTINUED

Function	Organism(s)	Agent(s)	Action/Response	Citation
Courtship/ reproduction	*Aplysia* sea hare	Egg laying hormone (ELH), ELH-related hormones	Affects reproductive behavior of neighboring individuals	Painter (1992)
	Penaeus vannamei Pacific white shrimp	Egg water	Induces gross morphological changes in sperm	Wang et al. (1995)
	Macrobrachium kistnensis freshwater prawn	Mucopolysaccharide from female sternal gland	Copulatory behaviour	Sarojini et al. (1982)
	Callinectes sapidus blue crab	Fraction of female urine smaller than ecdysone	Causes precopulatory behaviour	Gleeson et al. (1984)
	Rhithropanopeus harrisii crab	2–5 amino acid oligopeptides	Release of eggs, pumping response	Rittschof et al. (1990)
	Sea Lamprey	Testosterone	Prefer water with testosterone	Adams et al. (1987)
	Salmo salar Atlantic salmon	Testosterone	Electrical activity of olfactory nerve	Moore and Scott (1991)
	Fish	Specific steroids and prostaglandins	Stimulates milt production and coordinates spawning	Stacey et al. (1989)
Molting	*Daphnia pulicaria*	β-*N*-acetylglucosaminidase	Hydrolyzation of chitin	Vrba (1994)
	Alpheus heterochelis snapping shrimp	20-Hydroxyecdysone (exogenous)	Shortening of molt cycle by 65% at highest concentration	Mellon and Greer (1987)
Habitat conditioning	*Penaeus duorarum* pink shrimp	Protein precipitin	Precipitates proteins in solution	Smith (1987)
	Goldfish	Skin slime	Chelation of heavy metals	Allee (1951)

Alarm	*Anthopleura elegantissima* sea anemone	Molecule of 213.5 MW	Alarm	Howe and Sheikh (1975)
	Nassarius obsoletus mud snail	Molecule >100,000 MW from injured conspecific	Burial in substrate	Atema and Stenzler (1977)
	Calcinus laevimanus intertidal hermit crab	Water conditioned by deshelled conspecific	Increased locomotion in recipient	Hazlett (1990)
	Ostariophysan fishes	Molecule <500 MW, a pterin possibly isoxanthopterine from injured conspecific skin	Alarm	Pfeiffer and Lemke (1973)
	Abramis brama sea bream	Aqueous extract of skin	Defensive behavior, flight, hiding and stress	Malyukina et al. (1982)
Population regulation	*Lebistes reticulatus* guppy	Water-borne factor	Causes young guppies to be seen as food by older guppies	Rose (1959)
	Tilapia mossambica	High-molecular-weight antigen >8×10^5 daltons	Autoimmune reaction resulting in death	Henderson-Arzapalo et al. (1980)
	Rana pipiens leopard frog	Round, nonmotile cells 5–19 μm diameter with a heavy coat and large central vacuole	Inhibition of growth due to starvation	Richards (1958)
	Rana pipiens leopard frog	Water conditioned by conspecifics	Inhibits growth of small tadpoles	Rose (1960)

genic. *Penaeus duorarum* was found by Smith (1987) to secrete a substance that reacts with proteins in human blood. Smith claimed that these substances were produced in response to stress. However, he offered no controls to show that unstressed shrimp did not produce them. Nonetheless, he did show that shrimp under some circumstances release highly bioreactive substances into the water.

Crayfish. Information transmission via semiochemicals in crayfish has been studied both in static and flowing systems, with some debate over interpretation of the data. Some researchers hold that information concerning gender, stress, agonistic state, and population density can be transmitted downstream to a recipient crayfish (Rose, 1982; Hazlett, 1985). Others contend that such interpretation is questionable because of a problematic experimental design or statistical analysis (Itagaki and Thorp, 1981). However, much of the debate appears to be over the definition of "communication" versus "detection" (Thorp and Itagaki, 1982). From an aquacultural perspective, "detection" resulting in behavior that can be distinguished from normal behavior is sufficient as a mechanistic basis for potential enhancement or disruption of the production system—and the systems receiving the effluent.

Salmon. The ability of salmonids to home to natal waters, using ecomones as guides, is well documented (e.g., Hasler, 1966; Harden Jones, 1968). Aquaculturists have taken advantage of this remarkable phenomenon by formulating artificial ecomones to ensure that the salmon come back to a specific place for harvest (Hasler and Scholz, 1980). Researchers have found that the scent of other conspecifics, particularly juveniles, is a component of the bouquet of aromas constituting the right ecomone (Moore and Scott, 1991). Again, there is difficulty in defining this as communication, but it does result in a predictable, distinguishable behavior that can be manipulated for aquacultural purposes.

Minnows (and Their Relatives). One of the first known aquatic semiochemicals was named by Frisch (1941): *Schreckstoff* (fright substance). Schreckstoff is released from the skin of minnows and related fishes when specialized club cells are ruptured. The breaking of these club cells is facilitated by their being unusually fragile and the effect is to cause escape or other antipredation behavior in other members of the same or related species.

While overcrowding has been the focus of many studies, Allee (1951) has shown that undercrowding is inhibitory also. For goldfish, he demonstrated that maximal growth is not attained with minimum density, but at some greater, optimal density. He reasoned that the presence of other conspecifics conditions the water, probably by chelating or metabolizing toxins.

Catfish. Although the bullhead described in Todd's (1971) study is not the catfish that is typically cultured, it is congeneric with the species predominantly cultured in North America. In dealing with conspecifics, the brown bullhead (*Ictalurus nebulosus*) reportedly can identify gender by smell, associate water from an antagonist with the antagonist, detect changes when an antagonist has

been injured, and communicate with other individuals that conditions are too crowded to permit occupancy of territories.

Tilapia. A study of *Tilapia mossambica* in high-density culture revealed that a high-molecular-weight substance present in the slime layer caused anaphylactic shock and death in conspecifics once a certain density threshold was exceeded (Henderson-Arzapalo et al., 1980).

Tadpoles. Growth of *Rana pipiens* tadpoles under crowded conditions results in a size-differential semiochemistry, with the largest tadpoles (or even water from a separate tank containing large tadpoles) inhibiting the growth of the smaller tadpoles (Richards, 1958; Rose, 1960). Why the inhibitory effect is not expressed in the larger tadpoles is not known.

DISCUSSION

Aquatic animals respond to semiochemicals in ways that should be adaptive in their natural habitats. In nature, a dispersion pheromone may prevent exhaustion of local resources, spread of infectious diseases, inbreeding, and other negative consequences of overcrowding. An aggregation pheromone may alert conspecifics to availability of mates or a resource that could be shared. However, in an aquacultural setting, such semiochemical communication could be troublesome. For example, semiochemicals might inhibit growth or compromise immunocompetence when the animals are confined at high density, which is the norm for intensive aquaculture.

Chemical communication seems to have reached its evolutionary apex in the arthropods. Thus, intensive culture of arthropods such as shrimp and other crustaceans would seem especially vulnerable to problematic semiochemistry. We wonder if anticrowding semiochemicals might be involved in the chronic epidemics of viral and bacterial disease that have ravaged the farmed shrimp industry worldwide over the past decade (Lucien-Brun, 1997; Bedier et al., 1998).

If semiochemicals pose an internal problem for aquaculture, that problem logically becomes more insidious downstream. Because most intensive aquaculture relies on water exchange for diluting metabolites, the effluent carries any aquaculture-generated semiochemicals to the receiving body of water where the "message" is out of context. Semiochemicals used as attractants in the feed might attract indigenous animals to the effluent discharge point, where there would be no food. If the cultured animal is a predator, its natural prey downstream might be falsely warned of a danger that is not there and thus be repelled unnecessarily from the effluent discharge area. Animals raised at high densities might generate anticrowding semiochemicals. Relatively benign anticrowding semiochemicals might simply repel indigenous animals of the same or related species from the vicinity of the discharge point. More aggressive anticrowding semiochemicals might stunt growth, prevent molting, or impair the immune system.

While aggregating, alarm, stress, or anticrowding signals might confer some survival advantage within the aquaculture setting (and this is questionable), they would confer no survival advantage in the receiving system, because there would be no logical ecological mechanism for feedback to the signal-generating system. At best, any change in behavior on the part of the receiving organism in response to errant semiochemicals would not generate the evolutionarily expected benefit. Most probably, such a change in behavior would come at the expense of other behaviors that do convey a survival advantage (such as staying hidden). The logical outcome of uncoupling the generating and the receiving systems would be decreased production in the community of organisms living downstream, whether by a behavioral repulsion, decreased growth or growth efficiency, decreased immunocompetency, or by the nonadvantageous redistribution of conspecifics, congenerics, and their predators. These possible effects would constitute forms of endocrine disruption originating from natural biogenic sources, but geographically located and concentrated by human actions.

To what degree might semiochemicals from aquaculture actually disrupt wild populations or even entire aquatic communities downstream? We do not pretend to have an answer to this question, but we insist that it is worth asking. The answer will come only from carefully designed research that is yet to be performed. The beneficiaries of such research would be not only society and its natural resources, but also the aquaculture industry. This is because greatest adverse impacts of disruptive semiochemicals logically would be on the aquacultured organisms that are the source. If there is no problem, we all need to know that. But if there is a problem, then we need to know that too, so we can get on with a search for solutions.

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ATTRACTANT OR PHEROMONE: THE CASE OF NASONOV SECRETION AND HONEYBEE SWARMS

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Abstract—Honeybees are attracted to a variety of odors, including the secretion of their Nasonov glands, a secretion that has been widely assumed to be an orientation and attraction pheromone. A crossover design experiment comparing synthetic Nasonov secretion with linalool, oil of clove, skatole, and wax moth sex pheromone was established to determine if Nasonov secretion serves as a true pheromone or is simply a general attractant for honeybee swarms. None of the test odors was more attractive than odorless controls, and in all comparisons, synthetic Nasonov secretion was significantly more attractive than the test odors or odorless controls. The results confirm that Nasonov secretion is a true pheromone in the context of attracting honeybee swarms to nest cavities and that environmentally present or apparent odors play little or no role in honeybee nest-seeking behavior.

Key Words—*Apis mellifera*, linalool, skatole, clove, undecanal, nonanal, citral, geraniol, nerolic acid, geranic acid.

INTRODUCTION

A pheromone is a chemical signal released by an individual of a species for the purpose of eliciting in one or more other individuals of that species a response that is adaptive to both the releasing and receiving individuals. An attractant is simply a chemical that attracts an individual to it. An attractant need not benefit the individual attracted: indeed, it might benefit, harm, or be neutral to the attracted individual. A pheromone can be an attractant, but an attractant often is not a pheromone. Unless an attractant is emitted by a conspecific and designed to elicit a specific response from the receiver, it is not a pheromone—it might be a kairomone, a pheromone mimic, or some other odor present in the

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environment – but it is not a pheromone. To demonstrate that a chemical signal is truly a pheromone, we must be able to demonstrate that it is produced by one individual, is detected by a conspecific, and elicits a specific response in the receiving individual. The burden of proof that a chemical signal is a pheromone is rigorous and often difficult. In the case of attractants that are odors produced by the species of concern, first impressions might suggest that the odor is a pheromone, a conclusion that is not necessarily valid.

Nasonov secretion, a blend of six monoterpenes plus (E,E)-farnesol (Pickett et al., 1980) is widely regarded as the master honeybee attractant pheromone (Free, 1987). Its various postulated pheromonal roles include: attracting bees to form a cluster during the swarming process; marking the entrance of a new nest cavity or a new entrance to the existing hive, leading nestmates from the parent colony to the new nest cavity during swarming; marking and attracting nestmates to a queen that landed during the swarm flight; and scent-marking sources of water or sugar syrup that possess little odor. In general, Nasaonov secretion is considered to function to orient disoriented bees. We have used synthetic Nasonov pheromone as the key element in swarm traps that are highly attractive to honeybee swarms (Schmidt and Thoenes, 1987) and demonstrated that identical traps without pheromone attracted only 21% as many swarms as traps containing pheromone (Schmidt, 1994). These results suggested that Nasonov "pheromone" was, indeed, a pheromone that attracted other bees to potential nest sites. However, Wells et al. (1993) tested the assumption that Nasonov was a pheromone that attracted foragers to water or sugar sources. Of five tested odors, they discovered that cinnamon oil and cajeput oil when added to sugar syrup induced a larger increase in forager recruitment than the three Nasonov components, citral, geraniol, and nerol. After training to clove oil-scented syrup followed by syrup removal, foragers chose syrups in the order of oil of bay, anise odor, cajeput, clove oil, and finally Nasonov mixture (consisting of 1 : 1 : 0.5 citral, geraniol, and nerol). In a final test, bees were trained to syrup containing a blend of anise, bay, citral, geraniol, and nerol. On subsequent days when given a choice of the syrups containing individual scents, the order of choice by foragers was bay, geraniol, anise, and nerol, with citral being rejected. These data led Wells et al. (1993) to conclude that neither Nasonov secretion nor the components of the Nasonov secretion were a pheromone to attract bees to water or syrup. The authors suggested that in the situation of foragers at liquids, Nasonov secretion simply acted as an added odor to enable other foragers to find the source: it was not better than other odors; it simply was available as a means to impart an odor at the liquid source.

The work of Wells et al. (1993) raises doubts about the assumption that Nasonov secretion is a pheromone that governs nest-seeking and swarming behavior in honeybees. The goal of the research reported here was to test the null and alternative hypotheses: H_0 = Nasonov "pheromone" is simply an odor that enables

scout bees to detect potential nest cavities; H_a = Nasonov pheromone acts as a true attracting and orienting pheromone during the nest-seeking behavior.

METHODS AND MATERIALS

Swarm traps constructed of wood pulp were established in 20 locations within the Tucson, Arizona basin during 1997 and 1998. The traps had an internal volume of 31 liters and were in the shape of inverted truncated cones measuring 40 cm top diameter, 25 cm bottom diameter, and 40 cm high, each with a 3-cm-diameter hole at the bottom (Schmidt et al., 1989). Test odor lures consisted of: Nasonov mixture [1 : 1 : 1 (E) and (Z)-citral, geraniol, nerolic + geranic acids], linalool, skatole (3-methylindole), clove oil, and wax moth (*Galleria mellonella*) sex pheromone (3 : 1 nonanal: undecanal) (Dickens et al., 1986). The Nasonov mixture was selected as the maximally attractive Nasonov blend based on the work of Free et al. (1982). Those workers showed that the five terpenes blended in the 1 : 1 : 1 ratio were superior in attracting honeybees to cluster than all seven natural pheromone components [the five above, plus nerol and (E,E)-farnesol] combined in either equal proportions or in their natural ratios. Geraniol was obtained from Aldrich Chem. Co., Milwaukee, Wisconsin, nerolic + geranic acids from Bedoukian Research, Danbury, Connecticut, and the rest of the chemicals were from Sigma Chemical Co., St. Louis, Missouri. Each lure consisted of two 250-μl sealed polyethylene microtubes (Bio-Rad, Richmond, California) containing 100 μl each of the test material. The lures were then wrapped in black porous paper and attached with tacks inside the swarm traps just above the entrance hole. The rationale for the test odors was: Nasonov mixture is the putative pheromone; linalool is a highly attractive floral odor for bees; skatole likely would be present from the feces in the bottom of tree cavities inhabited by animals and it might be a deterrent odor; clove oil is a strong nonfloral plant odor; and wax moth sex pheromone provides an indication that a previous honeybee colony had sucessfully inhabited the cavity. These materials were selected to provide a wide chemical range of test odors.

A crossover test design was used to compare the attractiveness of the different odors. Half of the locations contained Nasonov pheromone lures in the traps and the other locations contained one of the test odors in the traps. Traps were attached at heights of 2–4 m to the trunks and main branches of mesquite, or other suitable trees. On each survey of the trap sites, the lures were reversed (i.e., sites with Nasonov received test odor, and sites with test odor received Nasonov). The number of swarms attracted to traps of each type was recorded. Swarm attraction was determined by the presence of a swarm inside a nest cavity when opened. Occupied swarm traps were replaced with new traps. Treatments were compared statistically using a chi-squared test, with Yates' correction, one degree of freedom (Snedecor, 1956).

TABLE 1. ODORS AS HONEYBEE SWARM ATTRACTANTS

Test odor	Swarms in traps with			
	Nasonov	Test odor	Nas/Test	Prob [a]
None[b]	19	4	4.75	0.002
Linalool	21	7	3.0	0.02
Skatole	26	4	6.5	0.001
Clove oil	17	2	8.5	0.001
Wax moth pheromone	9	1	9.0	0.05

[a]Chi-square test using Yate's correction.
[b]Data from Schmidt (1994).

RESULTS

Traps containing Nasonov "pheromone" attracted almost five times as many swarms as control traps lacking any added odor (Table 1). All four test odors also attracted many fewer swarms than the Nasonov pheromone and were statistically less attractive. There were no statistical differences between any of the four test odors and the no-odor control. Thus, none of the test odors was more attractive to swarms than no odor at all, and none was repellent to swarms. The only odor that actually attracted swarms was the synthetic Nasonov blend.

DISCUSSION

The four chosen odors—linalool, skatole, clove oil, and wax moth pheromone— represent a broad cross section of odors from floral, plant, and animal sources and from a species associated in nature with bee colonies. These odors might have biological relevance and are olfactorily apparent to honeybees. The finding that none of the test odors significantly attracted more (or fewer) honeybee swarms to nest cavities than odorless cavities indicates that general odor cues play little role in the nest discovery and acceptance process. It is possible that with larger sample sizes a statistical difference between test odors (for example, between linalool and wax moth pheromone) might be observed, but that difference almost certainly will be small compared to the effect of the Nasonov blend. Moreover, the purpose of this investigation was not to distinguish subtle differences between individual odors but to determine if any of them had a similar attractiveness to honeybee swarms as Nasonov secretion. Clearly none of the tested odors elicited attractive responses resembling that of Nasonov.

The results indicate that general source or environmental odors appear unimportant to bees in the nest-seeking process. This suggests that scout bees

initially are capable of finding nest cavities without the aid of odor and that, except in extreme situations, odor is not likely to be a factor involved in the process. The odors selected for testing are familiar odors that are easily detected by bees; thus, lack of response is not a consequence of inability to detect the odor. These findings may relate to efforts to discover repellents for honeybee swarms. Because odors appear not to affect swarm decisions pertaining to attraction to or acceptance of nest cavities, most odors placed in locations where bees are not desired are unlikely to be effective.

Water- or syrup-seeking and nest-site-seeking behaviors of honeybees are fundamentally different and are governed by different rules and sensory information. Water- and syrup-seeking behaviors involve individual foragers, plus their recruits, and are associated with short-term acquisition of water and energy. Most water and nectar or syrup sources are odorous. The actual odor itself of these sources appears less important than the fact that an odor exists to help the foragers locate the liquid source (Wenner and Wells, 1990). If no odor exists, foraging honeybees will add an odor– Nasonov. Nasonov is added not as a pheromone (Wells et al., 1993), but simply as the only, or most available, odor to the bees. There likely is no effective pheromone used by bees to mark liquid sources.

Nest site seeking behavior, unlike foraging behavior, is associated with a long-term, almost permanent, decision that literally affects the survival of the entire colony. Unlike a poor foraging decision, which might mean that the honeybee colony suffers a small potential loss of food or water, the cost of a decision to select a poor nest site is extreme. In this situation, which demands a "collective decision" of many bees to ensure a proper decision, communication is essential. A pheromonal system is an ideal and effective means for such communication. Honeybees appear to meet this communication need via their Nasonov pheromone. Nasonov secretion meets all the criteria necessary to be a pheromone—it is released by individuals to attract other individuals of the species to a specific location, the receivers respond by being attracted to the pheromone source, and the pheromonal response apparently is not elicited by other known odors or secretions.

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MAMMALIAN EXOCRINE SECRETIONS. XII: CONSTITUENTS OF INTERDIGITAL SECRETIONS OF BONTEBOK, *Damaliscus dorcas dorcas*, AND BLESBOK, *D. d. phillipsi*

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Abstract—In addition to the nine compounds identified in the interdigital secretion of the bontebok, *Damaliscus dorcas dorcas*, in a previous study, 76 compounds belonging to different compound types, were identified in the interdigital secretions of the bontebok and the blesbok, *D. d. phillipsi*. These compounds include alkanes, alcohols, aldehydes, ketones, fatty acids, terpenoids, γ-lactones, an isopropyl ester, long-chain hydroxyesters, 2-substituted pyridines, phenols, steroids, and dimethylsulfone. No qualitative differences were found between secretions from the two sexes or from animals from different habitats. Although no attempt was made to correlate territorial behavior or other behavioral phenomena with the qualitative composition of interdigital secretions from individual animals, available information seems to indicate that quantitative differences probably do not have a major semiochemical function. Only two species of bacteria, *Bacillus brevis* and *Planococcus citreus*, were found in the interdigital pouches of male and female members of the two subspecies, regardless of the habitat of the animals.

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B. brevis synthesized, among other unidentified constituents, (*Z*)-3-penten-2-ol, 2-hexanone, 2-octanone, 2-nonanone, tetradecanoic acid, pentadecanoic acid, heptadecanoic acid, octadecanoic acid, (*Z*)-9-hexadecenoic acid, and isopropyl hexadecanoate in vitro, while *P. citreus* produced, among others, the γ-lactones dodecan-4-olide and (*Z*)-6-dodecen-4-olide, which is one of the major constituents of the interdigital secretions of both subspecies. Some components of the interdigital secretions are not present in the interdigital glandular tissue, and the possibility is discused that these compounds could be produced by microbiological activity in the interdigital pouch.

Key Words—*Damaliscus dorcas*, mammalian semiochemicals, mammalian pheromones, exocrine secretions, interdigital secretions, chemical communication, mass spectrometry, NMR.

INTRODUCTION

Damaliscus dorcas is a member of the alcelaphine group of antelope (Family Bovidae, Tribe Alcelaphini) and is endemic to South Africa. The nominate subspecies *D. dorcas dorcas*, the bontebok, occupied a limited range on the coastal plains of the southwestern Cape Province. Populations were severely depleted and the subspecies narrowly averted extinction (Bigalke, 1955). Its status has improved considerably, although bontebok are still listed as rare in the South African Red Data Book (Smithers, 1986). The blesbok *D. dorcas phillipsi* was one of the dominant antelope of the central plateau grasslands of South Africa, and although it too suffered a considerable decline, it has been widely translocated and is now common and widespread on farms and reserves.

Both subspecies are of medium size (males approx. 60 kg). The bontebok has a more richly purplish brown body, with more white on the legs than the blesbok, and the large rump-patch is pure white. Both have $2n = 38$ chromosomes (Wurster and Benirschke, 1968), and they are capable of interbreeding (Fabricius et al., 1989). The social structure is similar to that of many African plains antelope, with territorial males, groups of bachelor males, and female (nursery or harem) herds. Blesbok also form mixed aggregations, mainly in winter (Lynch, 1974), but David (1973) did not find this to be the case in the bontebok population studied.

Some adult males establish territories that are most consistently occupied and defended around the time of the autumn rut in blesbok (Lynch, 1974) but occupied year-round by bontebok (David, 1973). Territorial advertising involves visual display, defecation on a limited number of dung sites on which animals often lie, and scent marking. Both sexes have preorbital glands, larger in males than in females, and interdigital or pedal glands on the forefeet. Lynch (1974) describes the interdigital gland secretion of blesbok as a yellow, odorous substance that adheres to the hairs between the digits. Territorial males occasionally

pawed their dung patches three or four times, either with one foot or alternately with both, when they returned to the patches, before lying down on them. David (1973) never observed bontebok pawing the ground in any context. Lynch presumed that blesbok males pawed to demarcate the territory but considered this an unimportant function since so little of the secretion could be rubbed off. The secretion adhering to the hair was thought to be more significant in marking the animal itself. Bigalke et al. (1980) tested the reaction of a captive male bontebok to interdigital secretion and its two major chemical components. The test substances were offered at a food trough on gauze swabs or in an airstream. Mean length of feeding bouts was not significantly affected. The test elicited irregular brief bouts of sniffing at the scent sources, which also occurred during some control runs. There were, in addition, a few shows of aggression, but these were also performed fairly regularly outside the test situation. The limited response to interdigital secretion, and the fact that its chemical composition shows little individual variation, was taken to indicate that the scent may only be species-specific. Spread about an inhabited area, it would merely indicate use by conspecifics and would not elicit particular reactions.

In two previous studies (Burger et al., 1976, 1977) only nine of the constituents of the complex interdigital secretion of the bontebok, *Damaliscus dorcas dorcas*, could be identified, largely due to the complexity of the secretion and a lack of expertise in this field of research. The evolution of capillary column technology and the accumulation of mass spectral data on the long-chain compounds that are typically found in mammalian exocrine secretions prompted us to reinvestigate this secretion. In this communication we wish to report the confirmation of the structures proposed for the previously identified compounds and the identification of an additional 77 constituents of the interdigital secretions of the bontebok and the related subspecies, *D. d. phillipsi*, the blesbok.

METHODS AND MATERIALS

General

All Pyrex glassware and the porcelain mortar and pestle used in the preparation and handling of biological material and extracts were heated to 500°C in an annealing oven to remove any traces of organic material. Dichloromethane (Merck, Residue Analysis Grade) was used for extraction purposes. Syringes, stainless-steel needles, and other apparatus were cleaned with this solvent.

Analytical Methods

Gas chromatographic (GC) analyses were carried out with a Carlo Erba 5300 gas chromatograph equipped with a flame ionization detector, Grob split-

splitless injector, and the following glass capillary columns: (1) 50 m × 0.3 mm, coated with PS-089 (polarity equivalent to SE-52) at a film thickness of 0.25 μm; (2) 40 m × 0.3 mm, coated with OV-1701-OH at a film thickness of 0.375 μm; and (3) 40 m × 0.25 mm coated with Superox 4 at a film thickness of 0.20 μm. All analyses were done with helium as carrier gas at a linear velocity of 28.6 cm/sec (column temperature 40°C). The flame ionization detector was operated at 280°C and the injector at 220°C. Samples were injected in the split mode, the analytes thermally focused on the column at ca. 30°C, and analyzed with a temperature program of 2°C/min from 40°C to 250°C (hold).

Electron impact (EI) mass spectra were recorded at 70 eV on a Carlo Erba QMD 1000 gas chromatograph—mass spectrometer (GC-MS system), with the columns and conditions described above. An interface temperature of 250°C was used. The ion source temperature was set at 180°C and the pressure in the source housing was ca. 2×10^{-5} torr at a column temperature of 40°C, decreasing to ca. 1×10^{-5} torr towards the end of the temperature program. Accurate mass measurements on synthetic reference compounds were done with a Varian MAT 311A high-resolution mass spectrometer and a Kratos DS 50 data system. ^{1}H and ^{13}C NMR spectra were recorded at 299.905 MHz and 75.42 MHz, respectively, at 25°C on a Varian VXR 300 NMR spectrometer.

Sample Collection and Preparation

Interdigital secretions were collected from bontebok captured in the Bontebok National Park, Swellendam, from a few blesbok captured in the Mountain Zebra National Park, Cradock, and from blesbok culled on the experimental farm of the University of Stellenbosch in the district of Heidelberg, Cape Province. Samples were taken from both sexes.

Surgical gauze squares (ca. 25 × 25 mm) consisting of several layers of surgical gauze were extracted for 5 hr with dichloromethane (Residue Analysis Grade), dried in an atmosphere of purified N_2 (activated charcoal), and stored in glass-stoppered bottles. Interdigital secretion was collected by rolling a gauze square around the tip of dressing forceps, inserting the forceps with gauze into the interdigital cavity, and collecting the secretion by rotating the forceps while removing it from the cavity. The gauze pads with the yellowish secretion were stored at −30°C in glass bottles with Teflon-lined screw caps until used for analysis.

Initially the secretion was extracted from the gauze with a minimum of dichloromethane in the smallest possible Soxhlet extractor. The problem with this method is that the extract has to be concentrated for further work by the evaporation of a considerable volume of dichloromethane, possibly resulting in the loss of some of the more volatile constituents of the secretion. The solvent was evaporated by placing the vial containing the extract in a 2-liter glass

beaker covered with aluminum foil and the solvent vapor purged from the beaker with purified N_2 (purified by activated charcoal) without blowing the purge gas directly into the vial containing the extract. Depending on the concentration of the extract and the size of the vial, the removal of 5 ml of dichloromethane took up to 10 hr.

To avoid the use of large volumes of solvent, the following method was also used: A glass vial containing dichloromethane (<5 ml) and the gauze pads on which the secretions of two animals had been collected were centrifuged for 1 min at 1500 rpm to improve contact between the gause and the small volume of solvent by compressing the gauze in the vial. The material in the vial was sonicated in the ultrasonic bath for 2 min, and the extract was separated from the gauze by centrifuging the gauze in a sintered glass filter insert suspended in a 5-ml Reacti-Vial. The extract was concentrated as described above. A comparison of this extract and an extract obtained by extracting any residual material from the gauze in a Soxhlet extractor showed that only negligible quantities of the carboxylic acids present in the secretion were left unextracted by this cold extraction method, and it was adopted for the extraction of the secretions of individual animals. Larger quantities of gauze were extracted in a Soxhlet extractor.

An interdigital gland was excised from the foreleg of a culled male bontebok. The gland, resembling a pouch with a depth of about 60 mm, a width of 20 mm and a wall thickness of about 3 mm, weighed 6.25 g. The few hairs on the inner glandular surface were removed with a razor blade and the gland was thoroughly washed with several quantities of pure dichloromethane, cut into small pieces, frozen with liquid N_2, and ground to a fine powder with a mortar and pestle cooled with liquid N_2. The glandular material was extracted with dichloromethane in a small Soxhlet extractor and the extract concentrated as described above. An extract of the interdigital gland of a blesbok was prepared in a similar manner.

Microbiological Experiments

The interdigital pouches of a male bontebok were swabbed with sterile gauze and the swabs placed into sterile McCartney bottles containing 0.89% saline solution. The samples were mixed thoroughly on a vortex mixer, after which 0.5 ml from each was enriched in Hutner's mineral salts medium (Bøvre and Hendriksen, 1976) containing 0.5% acetate as carbon source. The enrichments were incubated at 37°C for four days. The enriched cultures were vortexed and 10^{-1}–10^{-6} dilutions were spread-plated onto Hutner's mineral salts agar and incubated at 37°C for four days. Only two types of bacterial colonies were observed: white and yellow–orange. Several of these colonies were purified on Hutner's mineral salts agar, inoculated on Hutner's mineral salts agar

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slants, and incubated until visible growth was observed. These cultures were then subjected to phenotypic tests (Bøvre and Hendriksen, 1976; Cruickshank et al., 1975; Sneath et al., 1986). According to Sneath et al. (1986) the bacterial isolates could be classified as *Bacillus brevis* (white colony) and *Planococcus citreus* (yellow–orange colony).

The bacterial isolates were grown statically in 2 liters of liquid Hutner's mineral salts medium (Bøvre and Hendriksen, 1976) at 37°C for four days. After centrifugation, the supernatant containing the bacterial extracellular metabolites were decanted and concentrated from 2 liters to 15 ml under reduced pressure. The organic metabolic products were extracted with dichloromethane, but too little material was obtained for a satisfactory GC-MS analysis. From the bacteriological results, it could be seen that the bacterial isolates had a respirative metabolism requiring oxygen for rapid growth. The experiments were therefore repeated by supplementing Hutner's medium with 0.3% yeast extract and aerating the cultures during incubation. After dilution of the supernatants to facilitate proper extraction, the organic volatiles were extracted with dichloromethane in a liquid–liquid extractor. The dichloromethane extracts were carefully concentrated to 50 ml for GC-MS analysis by slow evaporation of the solvent at room temperature (see above). During concentration, the characteristic pleasant odor of (*Z*)-6-dodecen-4-olide was clearly detectable in the extract obtained from *P. citreus.*

Reference Compounds

Some of the compounds identified in the interdigital secretions of the bontebok and blesbok were available from previous research projects in this series, while others are commercially available from Merck (Darmstadt, Germany), Fluka (Buchs, Switzerland), Aldrich (Milwaukee, Wisconsin), Sigma (St. Louis, Missouri), and BASF (Ludwigshafen, Germany). The following compounds were synthesized during the present investigation. Boiling points are uncorrected.

4-Octanone. Condensation of butylmagnesium bromide with butanoyl chloride in tetrahydrofuran, hydrolysis of the resulting magnesium alcoholate with water, and conventional isolation procedures, gave 4-octanone (**11**) in 48% yield, bp 55–57°C (18 mm Hg). HR-MS: m/z $M^+ = 128.121$, calcd. for $C_8H_{16}O$ 128.120. ^1H NMR ($CDCl_3$): $\delta = 2.39$ (2H, t, $CH_3-CH_2-\mathbf{CH_2}-CO-$, $^3J_{H3,H2}$ 7.4 Hz), 2.38 (2H, t, $-CO-\mathbf{CH_2}-(CH_2)_2-$, $^3J_{H5,H6}$ 7.4 Hz), 1.2–1.7 (6H, m, $CH_3-\mathbf{CH_2}-CH_2-CO-$, $-CO-CH_2-(\mathbf{CH_2})_2-CH_3$), 0.91 (6H, t, CH_3, $^3J_{H1,H2} \approx {}^3J_{H8,H7}$ 7.1 Hz). ^{13}C NMR ($CDCl_3$: $\delta = 211.48$ (s, C-4), 44.77 (t, C-3), 42.60 (t, C-5), 26.08 (t, C-6), 22.46 (t, C-7), 17.40 (t, C-2), 13.88 (q, C-8), 13.79 (q, C-1).

4-Nonanone. Condensation of pentylmagnesium bromide and butanoyl chloride according to the general procedure described for 4-octanone, gave

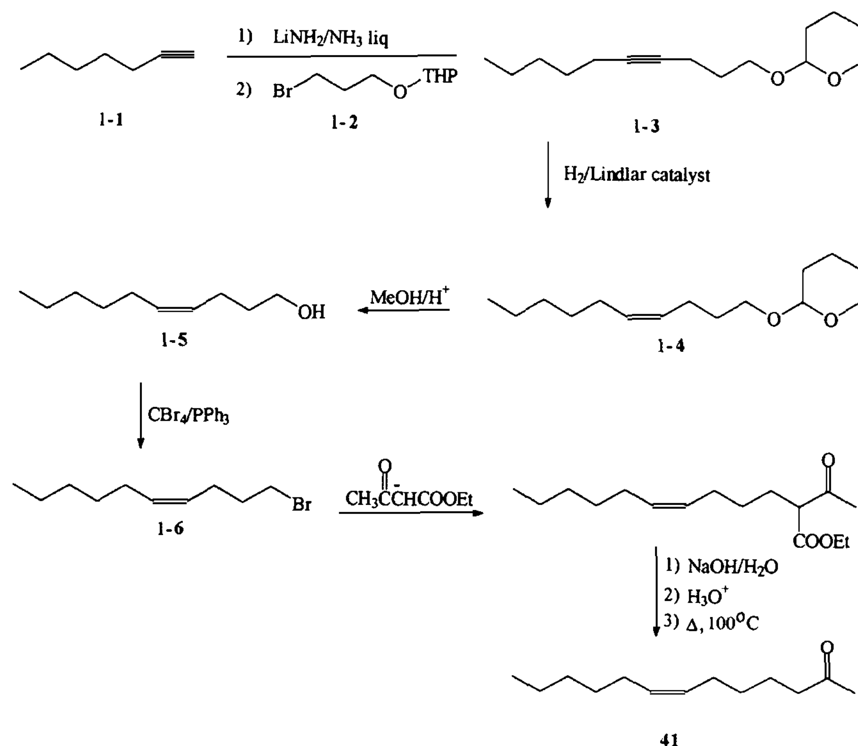
4-nonanone (**22**) in 50% yield, bp 75–77°C (20 mm Hg). HR-MS: m/z M^+ = 142.33, calcd. for $C_9H_{18}O$ 142.136. ^1H NMR ($CDCl_3$): δ = 2.38 (4H, t, —$\mathbf{CH_2}$—CO—$\mathbf{CH_2}$—), 1.2–1.7 (8H, m, CH_3—$\mathbf{CH_2}$—CH_2—CO—CH_2-($\mathbf{CH_2}$)$_3$—CH_3), 0.91 (6H, t, CH_3, $^3J_{H1,H2} \approx {}^3J_{H9,H8}$ 7.1 Hz). ^{13}C NMR ($CDCl_3$): δ = 211.69 (s, C-4), 44.78 (t, C-3), 42.87 (t, C-5), 31.67 (t, C-7), 23.76 (t, C-6), 22.48 (t, C-8), 17.48 (t, C-2), 13.81 (q, C-9), 13.70 (q, C-1).

7-Octen-2-one. Base-catalyzed condensation of 5-bromo-1-pentene with ethyl acetoacetate (Marvel and Hager, 1944), saponification of the condensation product, and decarboxylation of the corresponding β-ketoacid (Johnson and Hager, 1944) gave 7-octen-2-one (**13**) in 47% yield; bp 56–58°C (20 mm Hg). HR-MS: m/z M^+ = 126.102, calcd. for $C_8H_{14}O$ 126.104. ^1H NMR ($CDCl_3$): δ = 5.80 (1H, ddt, CHaHb=**CH**—, $^3J_{H7,H8a}$ 10.3 Hz, $^3J_{H7,H8b}$ 17.0 Hz), 5.00 (1H, ddt, CHa**Hb**=CH—, $^3J_{H8b,H8a}$ 2 Hz, $^3J_{H8b,H7}$ 17.0 Hz, $^4J_{H8b,H6}$ 1.4 Hz), 4.97 (1H, ddt, **CHa**Hb=CH—, $^2J_{H8b,H8a}$ 2 Hz, $^3J_{H8a,H7}$ 10.2 Hz), 2.43 (2H, t, CH_3—CO—$\mathbf{CH_2}$—, $^3J_{H4,H3} \approx {}^3J_{H4,H5}$ 7.4 Hz), 2.13 (3H, s, $\mathbf{CH_3}$—CO—), 2.06 (2H, ddt, CHaHb=CH—$\mathbf{CH_2}$—, $^3J_{H6,H5} \approx {}^3J_{H6,H7}$ 7.4 Hz, $^4J_{H6,H8b}$ 1.4 Hz), 1.60 (2H, quint. —CO—CH_2—$\mathbf{CH_2}$—, $^3J_{H4,H3} \approx {}^3J_{H4,H5}$ 7.4 Hz), 1.39 (2H, quint. —CO—(CH_2)$_2$—$\mathbf{CH_2}$—, $^3J_{H5,H4}$ 7.4 Hz). ^{13}C NMR ($CDCl_3$): δ = 209.0 (s, C-2), 138.5 (d, C-7), 114.7 (t, C-8), 43.6 (t, C-3), 33.5 (t, C-6), 29.8 (q, C-1), 28.4 (t, C-5), 23.3 (t, C-4).

*(Z)-7-Tridecen-2-one (**41**).* This was synthesized according to Scheme 1. 1-Heptyne (1-**1**) (20.22 g, 0.21 mol) was added dropwise to a suspension of lithium amide in liquid NH_3, prepared by allowing a solution of lithium (1.59 g, 0.23 mol) to react with liquid NH_3 in the presence of a catalytic quantity of Fe(III) nitrate. 1-Bromo-3-tetrahydropyranyloxypropane (1-**2**) (43.91 g, 0.20 mol) was added to the suspension of lithium amide over a period of 20 min, the reaction mixture was stirred for a further 1.5 hr, diluted with ether (300 ml), and left overnight to allow evaporation of the liquid NH_3. A mixture of NH_4Cl and $MgSO_4$ was added to the reaction mixture, the solids were removed by filtration, and the solvent evaporated to give 1-tetrahydropyranyloxy-4-decyne (1-**3**) in an undistilled yield of 91%. The product was used without purification for the preparation of the corresponding ethylenic compound (1-**4**).

A solution of the acetylenic compound (1-**3**) (43.12 g, 0.18 mol) in heptane/ethyl acetate (1 : 1, 106 ml) was partially hydrogenated in the presence of Lindlar catalyst (1.62 g, Fluka) and quinoline (1.5 ml) to give (Z)-1-tetrahydropyranyloxy-4-decene (1-**4**) in quantitative yield. The product was not distilled. MS: m/z (%) = 138(5), 110(3), 101(3), 95(3), 85(100), 84(22), 67(25), 55(33), 41(55).

Methanol was added slowly to a stirred suspension of (Z)-1-tetrahydropyranyloxy-4-decene (1-**4**) (43 g, 0.18 mol) in hydrochloric acid (2 M, 104 ml) until a homogenous solution was obtained. The solution was stirred at 40°C for 20 hr, and the largest part of the methanol was removed under reduced pressure.



SCHEME 1. Synthesis of (Z)-7-tridecen-2-one.

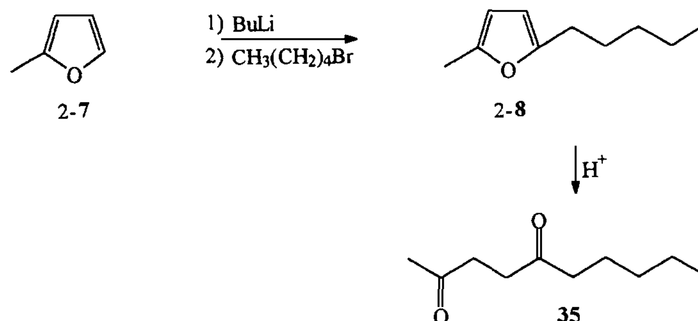
The residue was diluted with water (50 ml) and extracted with ether. The combined ether extracts were washed with $NaHCO_3$ solution, dried over $MgSO_4$, and the solvent evaporated to give (Z)-4-decen-1-ol (1-**5**) in a yield of 96%. MS: m/z (%) = 138(5), 110(9), 95(24), 81(77), 67(77), 55(75), 41(100).

Tetrabromomethane (36.81 g, 0.111 mol) was added to a solution of (Z)-4-decen-1-ol (1-**5**) (17.32 g, 0.111 mol) and triphenylphosphine (32.62 g, 0.124 mol) in dry acetonitrile (60 ml) at 0°C. The reaction mixture was stirred for 6 hr at room temperature, diluted with pentane (116 ml) and cooled to 5°C to allow the triphenylphosphine oxide to crystallize. The solid material was removed by filtration. The residual triphenylphosphine oxide was diluted with pentane, cooled to −30°C, and the triphenylphosphine oxide was removed. Evaporation of the solvent under reduced pressure and distillation of the residue gave (Z)-1-bromo-4-decene (1-**6**) in a yield of 85%; bp 107–108°C (5 mm Hg). MS: m/z (%) 220(26), 118(25), 192(1), 178(2), 176(2), 164(23), 162(23), 150(43), 148(43), 137(9), 135(20), 121(7), 119(5), 109(27), 107(13),

97(69), 95(30), 83(74), 81(77), 69(95), 67(76), 57(30), 55(100), 41(100), 39(96).

In a synthesis similar to that of 7-octen-2-one (**13**), the alkylation of ethyl acetoacetate (11.1 g, 85 mmol) with (Z)-1-bromo-4-decene (1-**6**) (20.71 g, 95 mmol), hydrolysis of the condensation product, and decarboxylation of the corresponding β-ketoacid gave (Z)-7-tridecen-2-one (**41**) in 43% yield; bp 132–134°C (4 mm Hg). HR-MS: m/z $M^+ = 196.181$, calcd. for $C_{13}H_{24}O$ 196.183. ^1H NMR ($CDCl_3$): $\delta = 5.35$ (4H, m, —**CH**=**CH**—, $^3J_{H7,H8}$ 10.8 Hz), 2.43 (2H, t, —CO—**CH**$_2$—, $^3J_{H4,H3} \approx {^3J_{H4,H5}}$ 7.6 Hz), 2.13 (1H, s, **CH**$_3$—CO—), 2.02 (4H, dtt, —**CH**$_2$—CH=CH—**CH**$_2$—, $^3J_{H6,H7} \approx {^3J_{H6,H5}} \approx {^3J_{H9,H8}} \approx {^3J_{H9,H10}}$ 7,5 Hz), 1.59 (2H, quint. —CO—CH$_2$—**CH**$_2$—, $^3J_{H4,H3} \approx {^3J_{H4,H5}}$ 7.6 Hz), 1.2–1.4 [8H, m, —**CH**$_2$—CH$_2$—CH=CH—CH$_2$—(**CH**$_2$)$_3$—], 0.89 (2H, t, **CH**$_3$—CH$_2$—, $^3J_{H13,H12}$ 6.9 Hz). ^{13}C NMR ($CDCl_3$): $\delta = 208.5$ (s, C-2), 130.4 (d, C-8), 129.1 (d, C-7), 43.7 (t, C-3), 31.6 (t, C-11), 29.8 (q, C-1), 29.4 (t, C-5*), 29.3 (t, C-10*), 27.2 (t, C-9), 26.9 (t, C-6), 23.5 (t, C-4), 22.6 (t, C-12), 14.1 (q, C-13) (*assignments interchangeable).

Decane-2,5-dione (35). This was synthesized according to Scheme 2. A solution of butyl lithium (0.30 mol) in tetrahydrofuran (140 ml) was cooled to −15°C and treated with 2-methylfuran (2-**7**). The resulting product, 2-lithio-5-methylfuran, was alkylated (Ramanathan and Levine, 1962; Büchi and Wüest, 1996) with 1-bromopentane (4.53 g, 0.30 mol) in tetrahydrofuran (48 ml) at −15°C. The reaction mixture was stirred at this temperature for 1 hr, left overnight at room temperature, and poured on ice. Conventional work-up procedures gave 5-methyl-2-pentylfuran (2-**8**) in a yield of 77%. ^1H NMR ($CDCl_3$): $\delta = 5.59$ (2H, d, =**CH**—**CH**=), 2.08 (3H, s, **CH**$_3$), 2.54 [2H, t, —**CH**$_2$—(CH$_2$)$_3$—CH$_3$], ca. 1.28 [6H, m, —CH$_2$—(**CH**$_2$)$_3$—CH$_3$], 0.98 [3H, t, —CH$_2$—(CH$_2$)$_3$—**CH**$_3$]. The product was used without purification for the preparation of decane-2,5-dione, in the following procedure.



SCHEME 2. Synthesis of decane-2,5-dione.

Dilute H_2SO_4 (1%, 19 ml) was added to a solution of 5-Methyl-2-pentylfuran (2-**8**) (35 g, 0.23 mol) in acetic acid (36 ml) and the reaction mixture was stirred at an oil bath temperature of 120°C for 3 hr (Büchi and Wüest, 1966). The reaction mixture was cooled to room temperature and poured into water. Isolation of the organic product in the normal manner gave a brown oil, which was fractionated to give decane-2,5-dione (**35**) in a yield of 78%; bp 77–78°C (0.1 mm Hg). HR-MS: m/z M^+ = 170.130, calcd. for $C_{10}H_{18}O_2$ 170.131. ^1H NMR ($CDCl_3$): δ = 2.69 (4H, m, CO—$\mathbf{CH_2}$—$\mathbf{CH_2}$—CO), 2.45 [2H, t, CO—$\mathbf{CH_2}$—$(CH_2)_3$—CH_3, $^3J_{H6,H7}$ 7.5 Hz], 2.18 (3H, s, $\mathbf{CH_3}$—CO), 1.58 [2H, quint. CO—CH_2—$\mathbf{CH_2}$—$(CH_2)_2$—, $^3J_{H7,H6} \approx {}^3J_{H7,H8}$ 7.4 Hz], 1.2–1.4 [4H, m, CO—CH_2—CH_2—$(\mathbf{CH_2})_2$—CH_3], 0.89 [3H, t, CO—$(CH_2)_4$—$\mathbf{CH_3}$, $^3J_{H10,H9}$ 6.9 Hz] ^{13}C NMR ($CDCl_3$): δ = 209.52 (s, C-5), 207.13 (s, C-2), 42.79 (t, C-6), 36.10 and 36.93 (t, C-3, C-4, assignment ambiguous), 31.42 (t, C-8), 29.89 (q, C-1), 23.57 (t, C-7), 22.48 (t, C-9), 13.91 (q, C-10).

Isopropyl Hexadecanoate (52). This was prepared by esterification of isopropyl alcohol and hexadecanoic acid with perchloric acid as catalyst, benzene as solvent, and azeotropic water removal. HR-MS: m/z M^+ = 298.286, calcd. for $C_{19}H_{38}O_2$ 298.287. ^{13}C NMR ($CDCl_3$) δ = 173.4 (s, C-1), 67.3 (d, C-1′), 34.8 (t, C-2), 32.0 (t, C-14), 29.2–29.7 (10t, C-4–C-13), 25.1 (t, C-3), 22.7 (t, C-15), 21.9 (2q, C-2′), 14.1 (q, C-16).

3-Propylphenol (31). This was prepared according to the method of Carvalho and Sargent (1984) with a yield of 52%; bp 114°C (20 mm Hg). HR-MS: m/z M^+ = 136.088, calcd. for $C_9H_{12}O$ 136.089. ^1H NMR ($CDCl_3$): δ = 7.12 (1H, m, =CR—CH=$\mathbf{CH}$—, $^3J_{H5,H6}$ 7.7 Hz, $^5J_{H5,H2}$ 0.85 Hz), 6.74 (1H, m, =CR—$\mathbf{CH}$=CH—, $^3J_{H4,H5}$ 7.6 Hz, $^4J_{H4,H6}$ 1.1 Hz, $^4J_{H4,H2}$ 1.6 Hz), 6.66 (1H, m, —$\mathbf{CH}$=CR—), 6.65 (1H, m, OH—C=$\mathbf{CH}$—, $^4J_{H6,H2}$ −2.7 Hz), 5.6 (1H, s, **OH**), 2.51 (2H, t, —$\mathbf{CH_2}$—CH_2—CH_3, $^3J_{H1',H2'}$ 7.6 Hz), 1.60 (2H, m, —CH_2—$\mathbf{CH_2}$—CH_3, $^3J_{H2',H1'}$, $^3J_{H2',H3'}$, 7.5 Hz), 0.91 (3H, t, $\mathbf{CH_3}$—). ^{13}C NMR ($CDCl_3$): δ = 155.31 (s, C-1), 129.44 (d, C-5), 121.20 (d, C-4), 115.59 (d, C-2), 112.71 (d, C-6), 144.76 (s, C-3), 37.95 (t, $\mathbf{CH_2}$—CH_2—CH_3), 24.35 (t, CH_2—$\mathbf{CH_2}$—CH_3), 13.79 (q, CH_2—CH_2—$\mathbf{CH_3}$).

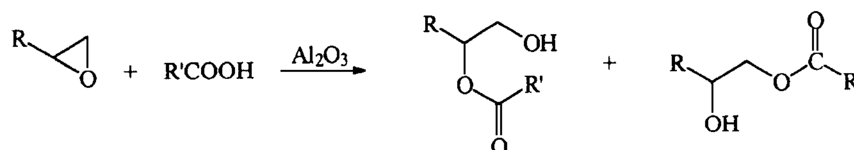
2-[(E)-4-Heptenyl]pyridine (37). 2-Picoline (10.99 g, 118 mmol) was added to a solution of phenyllithium (125 mmol) in ether (100 ml), and the mixture was stirred for 30 min at room temperature. (Z)-1-Chloro-3-hexene (7 g, 59 mmol) was added dropwise to the resulting solution of picolyllithium, and the reaction mixture was refluxed for 4 hr. A solution of ammonium chloride in dilute ammonia was added and the organic material isolated in the usual manner, with ether as a solvent. Fractionation of the organic product gave a mixture of isomeric pyridine derivatives in a yield of 70%, containing 7% 2-[(Z)-4-heptenyl]pyridine and 93% 2-[(*E*)-4-heptenyl]pyridine (GC); bp 82–86°C (5 mm Hg). HR-MS: m/z M^+ = 175.138, calcd. for $C_{12}H_{17}N$ 175.136. ^1H NMR ($CDCl_3$): δ = 8.5 (1H, ddd, $^5J_{H6,H3}$ 0.9 Hz,

$^3J_{H6,H5}$ 5.0 Hz, $^4J_{H6,H4}$ 1.8 Hz), 7.58 (1H, ddd, $^3J_{H4,H5}$ 7.7 Hz, $^3J_{H4,H3}$ 7.7, $^4J_{H4,H6}$ 1.8 Hz), 7.13 (1H, m, $^3J_{H3,H4}$ 7.7 Hz, $^4J_{H3,H5}$ 1.3 Hz, $^5J_{H3,H6}$ 0.9 Hz), 7.11 (1H, m, $^3J_{H5,H6}$ 5.0 Hz, $^3J_{H5,H4}$ 7.7 Hz, $^4J_{H5,H3}$ 1.3 Hz), 5.38 (2H, m, —**CH**=**CH**—, $^3J_{H4',H3'}$ 8.1 Hz, $^3J_{H4',H5'}$ 15.7 Hz, $^4J_{H4',H6'}$ 1.1 Hz, $^3J_{H5',H6'}$ 6.3 Hz), 2.80 [2H, t, $\mathbf{CH_2}$—$(CH_2)_2$—CH=CH—, $^3J_{H1',H2'}$, 7.8 Hz], 2.09 [2H, m, —$(CH_2)_2$—$\mathbf{CH_2}$—CH=CH—], 1.99 (2H, quint, —$\mathbf{CH_2}$—CH_2—CH=CH—CH_2—CH_3, $^3J_{H2',H1'}$ ≈ $^3J_{H2',H3'}$ 7.9 Hz), 1.79 (2H, m, —$\mathbf{CH_2}$—CH_3, $^3J_{H6',H7'}$ 7.5 Hz), 0.95 (3H, t, —CH_2—$\mathbf{CH_3}$, $^3J_{H7',H6'}$ 7.5 Hz). ^{13}C NMR ($CDCl_3$): δ = 162.21 (s, C-2), 149.15 (d, C-6), 136.22 (d, C-4), 132.17 (d, C4′), 128.53 (d, C-5′), 122.73 (d, C-3*), 120.91 (d, C-5*), 37.89 (t, C-2′), 29.91 (t, C-1′), 26.82 (t, C-3′), 20.57 (t, C-6′), 14.34 (q, C-7′). (*assignments interchangeable).

*2-Heptanoylpyridine (**42**).* A solution of 2-cyanopyridine (25.4 g, 0.24 mol) in ether (175 ml) was added over a period of 10 min to hexylmagnesium bromide (0.30 mol) in ether (100 ml) and the reaction mixture refluxed for 4 hr (Shaw et al., 1978). The practically black reaction mixture was carefully treated with cold water (25 ml) followed by dilute sulfuric acid (2.5 M, 300 ml) to give an orange solution. The ether was separated from the acidic aqueous phase containing most of the product and extracted twice with dilute sulfuric acid (1 M). The combined aqueous phases were heated on a steam bath for 15 min, cooled in an ice bath, and neutralized with Na_2CO_3. Extraction with ether and the usual isolation procedures gave 2-heptanoylpyridine (**42**) in a yield of 60%; bp 114–116°C (1 mm Hg). HR-MS: m/z M^+ = 191.131, calcd. for $C_{12}H_{17}NO$ 191.131. ^{1}H NMR ($CDCl_3$): δ = 8.68 (1H, ddd, $^3J_{H6,H5}$ 4.8 Hz, $^4J_{H6,H4}$ 1.7 Hz, $^5J_{H6,H3}$ 0.9 Hz), 8.04 (1H, ddd, $^3J_{H3,H4}$ 7.8 Hz, $^4J_{H3,H5}$ ≈ $^5J_{H3,H6}$ 1.1 Hz), 7.82 (1H, ddd, $^3J_{H4,H5}$ = 6.0 Hz, $^3J_{H4,H3}$ ≈ 7.5 Hz, $^4J_{H4,H6}$ 1.7 Hz), 7.46 (1H, ddd, $^3J_{H5,H4}$ 6.0 Hz, $^3J_{H5,H6}$ 4.8 Hz, $^4J_{H5,H3}$ 1.3 Hz), 3.22 (2H, t, —CO—$\mathbf{CH_2}$—CH_2—), 1.74 (2H, quint., —CO—CH_2—$\mathbf{CH_2}$—CH_2—), 1.4–1.2 [6H, m, —$(\mathbf{CH_2})_3$—CH_3], 0.89 [3H, t, —$(CH_2)_3$—$\mathbf{CH_3}$]. ^{13}C NMR ($CDCl_3$): δ = 202.13 (s, **CO**), 153.73 (s, C-2), 148.97 (d, C-6), 136.82 (d, C-4), 126.93 (d, C-5), 121.75 (d, C-3), 37.76 (t, CO—$\mathbf{CH_2}$—CH_2—), 31.78 (t, —$\mathbf{CH_2}$—CH_2—CH_3), 29.09 [t, CO—$(CH_2)_2$—$\mathbf{CH_2}$—], 24.03 (t, CO—CH_2—$\mathbf{CH_2}$) 22.61 (t, $\mathbf{CH_2}$—CH_3), 14.08 (q, —$\mathbf{CH_3}$).

1-Hydroxyalk-2-yl and 2-Hydroxyalk-1-yl Carboxylic Acid Esters. These were prepared by the Al_2O_3-catalyzed reaction of long-chain 1,2-epoxyalkanes with the appropriate carboxylic acids according to the general reaction scheme shown in Scheme 3.

A solution of butanoic acid (4.08 g, 46 mmol) in dry ether (10 ml) was added to Al_2O_3 (84.71 g, neutral, activity I) in dry ether (90 ml). The suspension was stirred for 15 min, treated with a solution of 1,2-epoxyoctadecane (3.04 g, 11 mmol) in ether (30 ml), and the reaction mixture stirred for a further 3 hr. After addition of CH_3OH (100 ml), the reaction mixture was stirred for 2 hr,



SCHEME 3. Synthesis of hydroxyesters.

the Al_2O_3 filtered off, and the filtrate concentrated on a rotary evaporator. The residue was taken up in ether and the solution washed with $NaHCO_3$ solution and water. Normal work-up methods gave a mixture of isomeric hydroxyesters in a yield of 76%. According to GC analysis on an apolar capillary column, the product contained 63% of 2-hydroxyoctadec-1-yl butanoate (**65**) and 37% of 1-hydroxyoctadec-2-yl butanoate (**64**). HR-MS (mixture of isomers): m/z M^+ = 356.328, calcd. for $C_{22}H_{44}O_3$ 356.329. NMR data were obtained from an analysis of the ^{1}H and ^{13}C spectra of the mixture of isomers. *Major component (**65**)*: ^{1}H NMR ($CDCl_3$): δ = 4.15 (1H, dd, CH**H**—O, $^2J_{H1'B,H1'A}$ −11.4 Hz, $^3J_{H1'B,H2'}$ 3.3 Hz), 3.96 (1H, dd, C**H**H—O, $^2J_{H1'A,H1'B}$ −11.4 Hz, $^3J_{H1'A,H2'}$ 7.3 Hz), 3.84 (1H, m, —**CH**—OH, $^3J_{H2',H3'}$ 6.3 Hz), 2.34 (2H, t, —**CH**$_2$—CO, $^3J_{H2,H3}$ 7.5 Hz), 1.89 (1H, s, O**H**), 1.67 (2H, m, CH_3—**CH**$_2$, $^3J_{H3,H4} \approx {}^3J_{H3,H2}$ 7.5 Hz), 1.2–1.6 [30H, m, CH_3—(**CH**$_2$)$_{15}$], 0.96 [3H, t, **CH**$_3$—(CH_2)$_2$, $^3J_{H4,H3}$ 7.5 Hz], 0.88 [3H, t, **CH**$_3$—(CH_2)$_{16}$, $^3J_{H18',H17'}$ 7.0 Hz]. ^{13}C NMR ($CDCl_3$): δ = 173.87 (s, C-1), 70.09 (d, C-2′), 68.55 (t, C-1′), 36.10 (t, C-2), 33.38 (t, C-3′), 31.94 (t, C-16′), 29.3–29.7 (11t, C-5′–C15′), 25.39 (t, C-4′), 22.70 (t, C-17′), 18.46 (t, C-3), 14.12 (q, C-18′), 13.67 (q, C-4). *Minor component (**64**)*: ^{1}H NMR ($CDCl_3$): δ = 4.92 (1H, m, **CH**—O, $^3J_{H1',H2'}$ 6.3 Hz), 3.72 (1H, dd, CH**H**—OH, $^2J_{H,H}$–12.0 Hz, $^3J_{H,H1'}$ 3.3 Hz), 3.62 (1H, dd, C**H**H—OH, $^2J_{H,H}$ −12.0 Hz, $^3J_{H,H1'}$ 6.3 Hz), 2.34 (2H, t, —**CH**$_2$—CO, $^3J_{H2,H3}$ 7.5 Hz), 1.89 (1H, s, O**H**), 1.67 (2H, m, CH_3—**CH**$_2$, $^3J_{H3,H4} \approx {}^3J_{H3,H2}$ 7.5 Hz), 1.2–1.6 [30H, m, CH_3—(**CH**$_2$)$_{15}$], 0.96 [3H, t, **CH**$_3$—(CH_2)$_2$, $^3J_{H4,H3}$ 7.5 Hz], 0.88 [3H, t, **CH**$_3$—(CH_2)$_{15}$, $^3J_{H17',H16'}$ 7.0 Hz]. ^{13}C NMR ($CDCl_3$): δ = 173.87 (s, C-1), 75.49 (d, C-1′), 64.99 (t, **CH**$_2$OH), 36.44 (t, C-2), 31.94 (t, C-15′), 30.55 (t, C-2′), 29.3–29.7 (11t, C-4′–C-14′), 25.38 (t, C-3′), 22.70 (t, C-16′), 18.46 (t, C-3), 14.12 (q, C-17′), 13.67 (q, C-4).

Treatment of 1,2-epoxyoctadecane (3.04 g, 11 mmol) with 2-methyl propanoic acid (4.08 g, 46 mmol) in ether and in the presence of Al_2O_3, as described in the foregoing synthesis of the hydroxyesters (**64** and **65**) gave 2.66 g (66%) of a mixture of 71% of 2-hydroxyoctadec-1-yl 2-methylpropanoate (**63**) and 29% of 1-hydroxyoctadec-2-yl 2-methyl propanoate. HR-MS (mixture of isomers): m/z M^+ = 356.332, calcd. for $C_{22}H_{44}O_3$ 356.329. *Major component (**63**)*: ^{1}H NMR ($CDCl_3$): δ = 4.14 (1H, dd, CH**H**—O, $^2J_{H1'B,H1'A}$ −11.3 Hz, $^3J_{H1'B,H2'}$ 3.2 Hz), 3.97 (1H, dd, C**H**H—O, $^2J_{H1'A,H1'B}$ −11.4 Hz, $^3J_{H1'A,H2'}$ 7.2 Hz), 3.83 (1H, m, $^3J_{H2',H3'}$ 6.4 Hz), 2.60 (1H, m, (CH_3)$_2$—**CH**—CO,

$^3J_{H2,H3} \approx {}^3J_{H2,CH_3}$ 14 Hz), 2.00 (1H, s, **OH**), 1.2–1.6 [30H, m, CH_3—$(\mathbf{CH_2})_{15}$], 1.85 [6H, m, $(\mathbf{CH_3})_2$—CH, $^4J_{H3,CH_3}$ −6.8 Hz, $^3J_{H3,H2} \approx {}^3J_{CH_3,H2}$ 13.6 Hz], 0.88 [3H, t, $\mathbf{CH_3}$—$(CH_2)_{16}$, $^3J_{H18',H17'}$ 6.7 Hz]. ^{13}C NMR ($CDCl_3$): δ = 177.47 (s, C-1), 70.11 (d, C-2′), 68.58 (t, C-1′), 33.95 (d, C-2), 33.37 (t, C-3′), 31.95 (t, C-16′), 29.3–29.7 (11t, C-5′–C-15′), 25.39 (t, C-4′), 22.71 (t, C-17′), 19.01 (q, C-3), 14.12 (q, C-18′). *Minor component* (1-hydroxyoctadec-2-yl 2-methylpropanoate): ^{1}H NMR ($CDCl_3$): δ = 4.89 (1H, m, **CH**—O, $^3J_{H1',H2'}$ 6.4 Hz), 3.75 (1H, dd, CH**H**—OH, $^3J_{H,H}$ −11.9 Hz, $^3J_{H,H1'}$ 3.4 Hz), 3.64 (1H, dd, **C**HH—OH, $^2J_{H,H}$ −11.9 Hz, $^3J_{H,H1'}$ 6.2 Hz), 2.58 [1H, m, $(CH_3)_2$—**CH**—CO, $^3J_{H2,H3} \approx {}^3J_{H2,CH_3}$ 14.0 Hz], 2.00 (1H, s, **OH**), 1.85 [6H, t, $(\mathbf{CH_3})_2$—CH, $^4J_{H3,CH_3}$ −6.8 Hz, $^3J_{H3,H2} \approx {}^3J_{CH_3,H2}$ 13.6 Hz], 1.2–1.6 [30H, m, CH_3—$(\mathbf{CH_2})_{15}$], 0.88 [3H, t, $\mathbf{CH_3}$—$(CH_2)_{15}$, $^3J_{H17',H16'}$ 6.7 Hz]. ^{13}C NMR ($CDCl_3$): δ = 177.47 (s, C-1), 75.40 (d, C-1′), 65.03 (t, $\mathbf{CH_2}OH$), 34.18 (d, C-2), 31.91 (t, C-15′), 30.53 (t, C-2′), 29.3–29.7 (11t, C-4′–C14′), 25.36 (t, C-3′), 22.69 (t, C-16′), 19.03 (q, C-3), 14.12 (q, C-17′).

A mixture of 1-hydroxyoctadec-2-yl pentanoate (**66**) (32%) and 2-hydroxyoctadec-1-yl pentanoate (**67**) (68%) was prepared from 1,2-epoxyoctadecane and pentanoic acid as described above. HR-MS (mixture of isomers): *m/z* M^+ = 370.347, calcd. for $C_{23}H_{46}O_3$ 370.345.

The reaction of 1,2-epoxynonadecane (3.0 g, 11 mmol) and butanoic acid (3.84 g, 44 mmol) in ether with Al_2O_3 as catalyst, gave (2.92 g, 74%) of a mixture of 2-hydroxynonadec-1-yl butanoate (**68**) (67%) and 1-hydroxynonadec-2-yl butanoate (33%). HR-MS (mixture of isomers): *m/z* M^+ 370.345, calcd. for $C_{23}H_{46}O_3$ 370.345. *Major component* (**68**): ^{1}H NMR ($CDCl_3$): δ = 4.16 (1H, dd, CH**H**—O, $^2J_{H1'B,H1'A}$ −11.3 Hz, $^3J_{H1'B,H2'}$ 3.0 Hz), 3.96 (1H, dd, **C**HH—O, $^2J_{H1'A,H1'B}$ −11.4 Hz, $^3J_{H1'A,H2'}$ 7.4 Hz), 3.85 (1H, m, —**CH**—OH, $^3J_{H2',H3'}$ 6.2 Hz), 2.34 (2H, t, —$\mathbf{CH_2}$—CO, $^3J_{H2,H3}$ 7.4 Hz), 1.73 (1H, s, **OH**), 1.67 (2H, m, CH_3—$\mathbf{CH_2}$, $^3J_{H3,H4} \approx {}^3J_{H3,H2}$ 7.4 Hz), 1.2–1.6 [32H, m, CH_3—$(\mathbf{CH_2})_{16}$], 0.96 [3H, t, $\mathbf{CH_3}$—$(CH_2)_2$, $^3J_{H4,H3}$ 7.4 Hz], 0.88 [3H, t, $\mathbf{CH_3}(CH_2)_{16}$, $^3J_{H19',H18'}$ 6.7 Hz]. ^{13}C NMR ($CDCl_3$): δ = 177.35 (s, C-1), 70.10 (d, C-2′), 68.56 (t, C-1′), 36.10 (t, C-2), 33.38 (t, C-3′), 31.94 (t, C-17′), 29.3–29.7 (12t, C-5′–C-16′), 25.38 (t, C-4′), 22.71 (t, C-18′), 18.46 (t, C-3), 14.12 (q, C-19′), 13.67 (q, C-4). *Minor component* (1-hydroxynonadec-2-yl butanoate): ^{1}H NMR ($CDCl_3$): δ = 4.92 (1H, m, **CH**—O, $^3J_{H1',H2'}$ 6.8 Hz), 3.72 (1H, dd, CH**H**—OH, $^2J_{H,H}$ −12.0 Hz, $^3J_{H,H1'}$ 3.0 Hz), 3.62 (1H, dd, **C**HH—OH, $^2J_{H,H}$ −12.0 Hz, $^3J_{H,H1'}$ 6.3 Hz), 2.33 (2H, t, —$\mathbf{CH_2}$—CO, $^3J_{H2,H3}$ 7.4 Hz), 1.73 (1H, s, **OH**), 1.67 (2H, m, CH_3—$\mathbf{CH_2}$, $^3J_{H3,H4} \approx {}^3J_{H3,H2}$ 7.4 Hz), 1.2–1.6 [32H, m, CH_3—$(\mathbf{CH_2})_{16}$], 0.96 [3H, t, $\mathbf{CH_3}$—$(CH_2)_2$, $^3J_{H4,H3}$ 7.4 Hz], 0.88 [3H, t, $\mathbf{CH_3}$—$(CH_2)_{16}$, $^3J_{H18',H17'}$ 6.7 Hz]. ^{13}C NMR ($CDCl_3$): δ = 177.35 (s, C-1), 75.43 (d, C-1′), 64.93 (t, $\mathbf{CH_2}OH$), 36.30 (t, C-2), 31.94 (t, C-16′), 30.55 (t, C-2′), 29.3–29.7 (12t, C-4′–C-15′), 25.38 (t, C-3′), 22.71 (t, C-17′), 18.46 (t, C-3), 14.12 (q, C-18′), 13.67 (q, C-4).

RESULTS AND DISCUSSION

A typical total ion chromatogram of an extract of the interdigital secretion of a male bontebok is shown in Figure 1. The secretions of male and female members of both subspecies were qualitatively identical, regardless of the habitat of the animals. Many of the constituents of the interdigital secretions were tentatively identified by comparison of their low-resolution electron impact mass spectra with those in NBS and Wiley mass spectra libraries. Further diagnostic information was obtained from the chemical ionization mass spectra of some of the constituents. The electron impact mass spectra of certain long-chain compound classes contain so little information in the higher mass ranges that it is impossible to detect certain types of chain branching. The structures of the majority of the compounds identified were therefore confirmed by gas chromatographic comparison (coelution) with authentic commercially available or synthesized material. Some compound classes were represented by several members of the respective homologous series of compounds, in which case representative compounds only were synthesized for comparison. The compounds identified in the interdigital secretions are listed in Table 1 together with the relevant mass spectral data, information on the analytical techniques employed in their identification, and some quantitative data on the major constituents present in the secretions in quantities higher than 1 ng/animal.

Members of the homologous series of alkanes, 1-alkanols, 2-alkanols, alkanals, 2-, 3- and 4-alkanones, and alkanoic acids were all found to have unbranched structures. Of these constituents (*Z*)-3-penten-2-ol, 2-nonanol, pentanal, (*E*)-2-hexenal, (*E*)-2-nonenal, (2Z,4Z)-2,4-heptadienal, and (2Z,4Z)-2,4-decadienal were detected and identified with a capillary column coated with OV-1701-OH. 2-Decanone was detected by using a capillary column coated with Superox 4. The secretions contain several terpenoid compounds, of which squalene had previously been identified in the dorsal secretion of the springbok, *Antidorcas marsupialis* (Burger et al., 1981). In addition to the diketone, undecane-2,5-dione, identified in the secretion of the bontebok in a previous study (Burger et al., 1976), decane-2,5-dione was found in the secretions in the present study.

The interdigital secretions of the two subspecies also contain various long-chain hydroxyesters of the type previously identified in the dorsal secretion of the springbok, *Antidorcas marsupialis* (Burger et al., 1981) and in the preorbital secretion of the grysbok, *Raphicerus melanotis* (Burger et al., 1996), and steenbok, *R. campestris* (Burger et al., unpublished results). So far no information is available on the stereochemistry and possible function of long-chain hydroxyesters in the secretions in which they have been identified. These aspects have not been investigated in the present study and will be subjected to further investigation. Phenol, 3-methylphenol, 3-ethylphenol, and 3-propylphenol are responsible for the faint but distinctive cresollike smell of the secretions

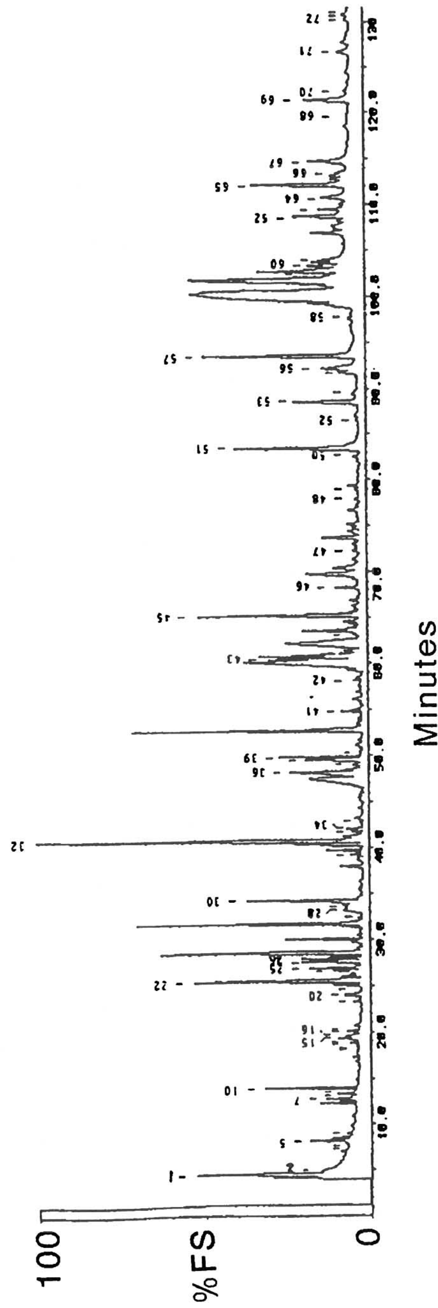


FIG. 1. Total ion chromatogram of an extract of the interdigital secretion of a male bontebok, *Damaliscus dorcas dorcas*. Gas chromatographic conditions as given in the experimental part.

TABLE 1. COMPOUNDS IDENTIFIED IN INTERDIGITAL SECRETION OF BONTEBOK AND BLESBOK

No. in Figure 1	Compounds	Quantity (ng/animal)[a]				EI mass spectral data [m/z (%)]
		Bontebok		Blesbok		
		Male	Female	Male	Female	
	Alkanes					
4	Octane[b,c]					114(7), 85(40), 71(30), 57(44), 43(100), 41(40)
10	Nonane[b,c]	35	2	1	1	128(8), 99(8), 85(35), 71(28), 57(87), 43(100), 41(55)
40	Tetradecane[b,c]			3	1	198(4), 155(1), 141(3), 127(3), 113(5), 99(10), 85(36), 71(55), 57(100), 43(81), 41(50)
44	Hexadecane[b,c]			7		226(4), 169(1), 155(1), 141(3), 127(3), 113(5), 99(10), 85(36), 71(55), 57(100), 43(81), 41(55)
	Terpenoids					
21	γ-Terpinene[b,c]					136(30), 121(32), 105(11), 93(100), 91(56), 79(27), 77(44), 43(20), 41(24)
26	Linalool[b,c]	33	2			136(18), 121(25), 93(100), 91(41), 80(21), 79(30), 77(27), 71(35), 69(38), 67(28), 55(24), 43(43), 41(72)
28	*p*-Cymen-α-ol[b,c]	1	3	13	7	150(10), 135(79), 91(28), 65(10), 43(100)
30	α-Terpineol[b,c,d]	80	14	66	8	136(45), 121(45), 107(7), 93(60), 81(45), 73(28), 67(26), 60(35), 59(100), 43(67)
72	Squalene[b,c]					175(1), 161(1), 149(4), 136(6), 121(8), 107(6), 95(12), 81(46), 69(100), 55(4), 41(23)
	Alcohols					
	(*Z*)-3-Penten-2-ol[b,c,e]					86(6), 71(100), 58(4), 53(15), 45(36), 43(90), 41(46)
6	2-Hexanol[b,c]	5				87(6), 71(5), 69(9), 45(100), 43(20), 41(18)
20	1-Heptanol[b,c]					98(2), 83(5), 70(95), 56(100), 41(89)

	2-Nonanol[b,c,e]					129(3), 98(4), 69(8), 57(18), 56(8), 55(5), 45(100), 43(25), 41(14)
49	1-Hexadecanol[b,c]					196(1), 168(1), 154(1), 140(2), 125(5), 111(18), 97(50), 83(70), 69(75), 57(70), 55(100), 43(85), 41(70)
54	1-Octadecanol[b,c]					111(18), 97(33), 83(61), 69(38), 57(73), 55(67), 43(94), 41(100)
58	1-Icosanol[b,c]					167(3), 153(2), 139(4), 125(10), 111(24), 97(46), 83(57), 69(55), 57(88), 55(70), 43(100), 41(70)
61	1-Henicosanol[b,c]	18	5			125(18), 111(35), 97(75), 83(85), 69(75), 57(100), 55(99), 43(90), 41(85)
62	1-Docosanol[b,c]	55	14	8	19	181(1), 167(2), 153(3), 139(5), 125(16), 111(37), 97(65), 83(76), 69(60), 57(70), 55(75), 43(100), 41(69)
69	1-Tetracosanol[b,c]	82	6	17	33	181(2), 167(4), 153(4), 139(7), 125(5), 111(29), 97(63), 83(76), 69(59), 57(78), 55(75), 43(100), 41(65)
75	1-Hexacosanol[b,c]					181(4), 167(5), 153(5), 139(6), 125(14), 111(33), 97(48), 83(55), 69(48), 57(85), 55(65), 43(100), 41(61)
	Aldehydes					
	Pentanal[b,c,e]					86(12), 71(5), 58(33), 57(25), 44(100), 43(92), 42(18), 41(50)
5	Hexanal[b,c,f]	13	11		7	82(14), 72(23), 67(13), 57(63), 56(89), 44(100), 43(65), 41(95)
	(*E*)-2-Hexenal[b,c,e]					98(18), 83(43), 80(13), 70(18), 69(49), 57(40), 55(74), 41(100)
8	Heptanal[b,c,f]	11	5	4	5	96(10), 86(15), 81(20), 71(24), 70(90), 57(62), 55(65), 44(100), 43(78), 42(60), 41(94)
15	(*E*)-2-Heptenal[b,c,f]					112(7), 111(2), 97(10), 94(1), 84(12), 83(69), 70(38), 69(39), 68(35), 57(54), 56(48), 55(68), 41(100)
	(2*Z*,4*Z*)-2,4-Heptadienal[b,c,e]					110(20), 95(10), 81(100), 67(21), 53(30), 41(33)

TABLE 1. CONTINUED

No. in Figure 1	Compounds	Quantity (ng/animal)[a] Bontebok Male	Bontebok Female	Blesbok Male	Blesbok Female	EI mass spectral data [m/z (%)]
18	Octanal[b,c]	5	4	14	3	110(5), 100(12), 85(19), 84(50), 69(38), 57(78), 56(73), 44(80), 43(100), 42(40), 41(97)
19	(*E*)-2-Octenal[b,c,f]	3	3	2	3	111(4), 98(8), 97(10), 83(40), 70(58), 69(30), 57(65), 55(100)
	(*E*)-2-Nonenal[b,c,e]					122(6), 112(3), 111(8), 97(11), 96(15), 83(48), 70(68), 69(52), 57(50), 55(93), 43(100), 41(60)
	(2*Z*,4*Z*)-2,4-Decadienal[b,c,e,f]					152(10), 123(6), 95(17), 83(13), 81(100), 67(27), 55(35), 53(22), 41(60)
	Ketones					
1	2-Pentanone[b,c,f]	24	19	16	4	86(12), 71(34), 57(9), 43(100), 41(18)
2	3-Methyl-2-butanone[b,c]					86(13), 71(4), 43(100), 41(18)
3	2-Hexanone[b,c,f]	2	1			100(10), 85(7), 71(5), 58(65), 43(100), 41(15)
7	2-Heptanone[b,c,f,g]	17	4	3	3	114(4), 99(3), 85(3), 71(12), 59(12), 58(63), 43(100)
11	4-Octanone[b,c,f]					128(33), 99(5), 85(80), 71(100), 58(65), 57(93), 43(87), 41(65)
13	7-Octen-2-one[b,c]	3	3	1	2	126(3), 111(2), 108(3), 99(10), 97(5), 83(3), 71(19), 68(16), 58(4), 55(9), 43(100), 41(25)
14	3-Octanone[b,c,f]	1	8	1	4	128(5), 99(39), 85(8), 72(58), 71(48), 57(62), 43(100), 41(15)
16	2-Octanone[b,c,f]	16	8	10	6	128(4), 113(2), 85(5), 71(16), 59(14), 58(65), 43(100)
22	4-Nonanone[b,c,f]	86		2	9	142(4), 99(75), 86(37), 71(100), 58(68), 55(22), 43(95), 41(55)

25	2-Nonanone[b,c,f,g]	29	13	11	12	142(3), 127(3), 113(1), 99(2), 85(5), 71(46), 59(22), 58(96), 43(100), 41(15)
	2-Decanone[b,c,h]					156(4), 85(6), 71(30), 59(37), 58(99), 43(100), 41(22)
32	(*Z*)-5-Undecen-2-one[b,c,g]	48	23	70	101	168(2), 150(2), 139(2), 125(5), 110(26), 97(20), 81(44), 68(33), 58(15), 54(43), 43(100), 41(55)
33	(*Z*)-7-Undecen-2-one[b]	5	1	1	1	150(1), 125(6), 110(8), 97(6), 81(14), 71(17), 69(15), 58(9), 55(16), 43(100), 41(33)
34	2-Undecanone[b,c,f,g]	8	1	3	3	170(2), 127(2), 112(3), 110(3), 85(8), 71(30), 59(28), 58(100), 43(80), 41(20)
35	Decane-2,5-dione[b,c]	1	1	1	1	127(14), 114(36), 99(54), 71(70), 43(100)
39	Undecane-2,5-dione[b,c,f,g]	46	36	7	10	141(3), 127(4), 114(44), 99(38), 85(15), 71(60), 43(100), 41(20)
41	(*Z*)-7-Tridecen-2-one[b,c,f]	12	1	3	7	178(2), 138(7), 125(9), 110(10), 97(14), 96(15), 81(23), 71(39), 67(25), 58(9), 55(26), 54(20), 43(100), 41(38)
	Carboxylic acids					
17	Hexanoic acid[b,c]	62	1	6	2	87(15), 73(50), 60(100), 55(20), 43(35), 41(45)
24	Heptanoic acid[b,c]	11	6	6	5	101(6), 87(22), 73(43), 60(100), 55(36), 43(50), 41(64)
29	Octanoic acid[b,c]					115(8), 101(22), 85(20), 73(73), 60(100), 55(37), 43(48), 41(38)
36	Decanoic acid[b,c]	51	17	21	32	172(3), 154(2), 143(7), 129(40), 115(12), 101(6), 87(15), 73(92), 71(40), 60(100), 57(42), 55(62), 43(65), 41(77)
43	Dodecanoic acid[b,c,f]	89	61		22	200(20), 171(1), 157(20), 143(6), 129(38), 115(10), 87(18), 73(96), 60(100), 57(47), 55(47), 43(78), 41(70)
47	Tetradecanoic acid[b,c,f]	6	2	2	3	228(17), 185(22), 171(12), 157(7), 143(14), 129(38), 115(14), 97(17), 85(36), 73(90), 71(35), 69(35), 60(68), 57(55), 55(54), 43(100), 41(91)

TABLE 1. CONTINUED

No. in Figure 1	Compounds	Quantity (ng/animal)[a]				EI mass spectral data [m/z (%)]
		Bontebok		Blesbok		
		Male	Female	Male	Female	
48	Pentadecanoic acid[b,c]	3	1	3	1	242(12), 213(2), 199(12), 185(8), 171(7), 157(8), 143(15), 129(30), 115(10), 97(18), 83(28), 73(83), 71(37), 69(39), 60(60), 57(62), 55(60), 43(100), 41(92)
50	(Z)-9-Hexadecenoic acid[b,c]					236(2), 194(1), 151(1), 138(3), 123(8), 111(14), 97(35), 83(47), 69(84), 55(100), 41(90)
51	Hexadecanoic acid[b,c,f]	127	42	66	92	256(17), 227(3), 213(15), 199(4), 185(9), 171(11), 157(13), 143(6), 129(32), 115(11), 97(19), 83(23), 73(80), 60(61), 57(57), 55(57), 43(100), 41(85)
53	Heptadecanoic acid[b,c]	59	15	29	31	270(4), 241(3), 227(13), 213(3), 199(2), 185(11), 171(12), 157(3), 143(4), 129(35), 115(15), 97(23), 85(28), 83(29), 73(70), 60(55), 57(59), 55(56), 43(100), 41(83)
55	(9Z,12Z)-9,12-Octadecadienoic acid[b,c]	12	19	9	48	280(5), 196(1), 164(2), 150(5), 136(8), 123(15), 109(34), 95(68), 81(88), 67(100), 55(89), 43(58), 41(89)
56	(Z)-9-Octadecenoic acid[b,c]	25	5	8	32	264(5), 222(2), 180(2), 165(2), 137(4), 123(9), 111(13), 97(36), 83(45), 81(35), 69(72), 67(40), 55(100), 43(73), 41(80)
57	Octadecanoic acid[b,c,f]	156	56	67	87	284(5), 255(3), 241(18), 227(4), 213(2), 199(5), 185(15), 171(6), 157(3), 143(4), 129(39), 115(12), 101(7), 97(27), 85(28), 73(100), 60(88), 55(87), 43(95), 41(68)
59	Icosanoic acid[b,c,f]					312(15), 269(5), 255(3), 241(2), 227(3), 213(2), 199(3), 185(7), 171(4), 157(4), 143(3), 129(38), 115(14), 101(6), 97(27), 85(21), 73(100), 60(73), 55(58), 43(96), 41(60)

	Lactones					
45	(Z)-6-Dodecen-4-olide[b,c,d,f]	17	6	3	4	196(1), 136(4), 121(2), 105(1), 96(8), 85(100), 79(8), 67(8), 55(10), 41(17)
46	Dodecan-5-olide[b,c,f]	129	96	70	75	155(3), 136(4), 114(10), 99(100), 83(8), 71(44), 70(30), 55(45), 43(34), 42(31), 41(34)
	Ester					
52	Isopropyl hexadecanoate[b,c,f]					257(14), 256(19), 239(12), 213(5), 199(2), 185(4), 171(4), 157(5), 143(3), 129(15), 111(7), 102(44), 97(15), 83(20), 73(34), 71(31), 69(29), 60(67), 57(55), 55(48), 43(100), 41(50)
	Hydroxyesters					
60	2-Hydroxyoctadec-1-yl ethanoate[b]	23	9	2	6	255(3), 125(4), 111(10), 103(11), 97(18), 83(19), 74(32), 69(16), 57(22), 55(20), 43(100)
63	2-Hydroxyoctadec-1-yl 2-methylpropanoate[b,c]	21	8	3	21	255(3), 131(7), 111(5), 102(38), 87(24), 71(100), 57(27), 55(27), 43(56)
64	1-Hydroxyoctadec-2-yl butanoate[b,c]	35	9	4	15	255(3), 131(5), 111(4), 102(17), 87(15), 71(100), 57(23), 55(18), 43(36)
65	2-Hydroxyoctadec-1-yl butanoate[b,c]	101	39	7	61	255(3), 131(6), 111(5), 102(27), 87(24), 71(100), 57(20), 55(20), 43(41)
66	1-Hydroxyoctadec-2-yl pentanoate[b,c]	10	8	4	9	255(1), 145(3), 116(15), 101(20), 85(100), 69(20), 57(90), 43(42)
67	2-Hydroxyoctadec-1-yl pentanoate[b,c]	37	36	13	26	255(2), 145(5), 116(30), 101(41), 85(100), 69(20), 57(100), 43(34)
68	2-Hydroxynonadec-1-yl butanoate[b,c]					269(2), 131(4), 111(5), 102(25), 87(19), 71(100), 57(23), 55(25), 43(52)
70	2-Hydroxyicos-1-yl 2-methylpropanoate[b]					283(1), 131(5), 111(5), 102(28), 87(16), 71(100), 57(40), 55(25), 43(67)
71	2-Hydroxyicos-1-yl butanoate[b]	27	8	2	6	283(2), 131(6), 111(7), 102(23), 87(20), 71(100), 57(29), 55(28), 43(46)
73	2-Hydroxyicos-1-yl pentanoate[b]	10	5	3	3	283(1), 145(5), 116(28), 101(37), 85(100), 69(19), 57(100), 43(32)

TABLE 1. CONTINUED

No. in Figure 1	Compounds	Quantity (ng/animal)[a] Bontebok Male	Bontebok Female	Blesbok Male	Blesbok Female	EI mass spectral data [m/z (%)]
74	2-Hydroxyhenicos-1-yl butanoate[b]					297(1), 131(5), 111(6), 102(24), 87(20), 71(100), 57(38), 55(27), 43(39)
76	2-Hydroxydocos-1-yl butanoate[b]					311(1), 131(6), 111(8), 102(25), 87(19), 71(100), 57(42), 55(35), 43(50)
	Pyridines					
37	2-[(*E*)-4-Hepten-1-yl] pyridine[b,c,f]	4	5	2	1	160(6), 146(4), 133(10), 118(6), 106(14), 93(100), 78(6), 65(6), 51(5), 41(7)
38	2-Heptylpyridine[b,c,d,f]	32	48	22	16	177(1), 148(4), 134(5), 120(16), 106(25), 93(100), 78(6), 65(5), 51(3), 41(6)
42	2-Heptanoylpyridine[b,c]	5	4	1	1	191(8), 163(7), 148(14), 134(48), 120(27), 106(42), 93(19), 79(100), 78(78), 51(17), 43(21), 41(21)
	Phenols					
12	Phenol[b,c]					94(100), 66(40), 65(28), 55(4)
23	3-Methylphenol[b,c,d]	59	42	5	124	108(94), 107(100), 90(15), 80(15), 79(46), 77(46), 65(5), 63(8), 53(13), 51(15), 50(10)

27	3-Ethylphenol[b,c]	2		5	6	122(30), 108(9), 107(100), 91(7), 77(21), 65(5), 53(4)
31	3-Propylphenol[b,c]	7	3	27	2	136(56), 121(22), 108(59), 107(100), 98(19), 94(12), 91(16), 78(19), 77(40), 65(13), 51(9)
	Steroids					
	Cholesterol[b,c,i]					386(18), 368(13), 353(15), 326(4), 301(23), 275(31), 255(19), 231(14), 213(34), 199(14), 185(12), 173(18), 159(37), 145(58), 133(38), 119(42), 105(71), 95(64), 81(73), 67(52), 55(82), 43(100)
	Desmosterol[b,c,i]					384(5), 369(8), 351(6), 300(8), 271(37), 253(12), 213(15), 199(7), 185(7), 173(9), 159(22), 145(28), 133(22), 119(21), 105(41), 95(42), 81(42), 69(100), 55(58), 41(60)
	Other					
9	Dimethylsulfone[b,c,f]					94(61), 79(100), 65(3), 64(4), 63(7), 48(8), 45(25)

[a]Quantities lower than 1 ng/animal not given.
[b]Low-resolution mass spectrum.
[c]Retention-time comparison with authentic synthetic material.
[d]Identified in previous study (Burger et al., 1977).
[e]Detected using OV-1701-OH column.
[f]Low-resolution CI(CH_4)-MS.
[g]Identified in previous study (Burger et al., 1976).
[h]Detected using Superox 4 column.
[i]Elutes beyond retention time range shown in Figure 1.

to the human nose. Although aromatic compounds are fairly common in mammalian exocrine secretions, the pyridine derivatives 2-heptylpyridine, 2-[(*E*)-4-heptenyl)]pyridine, and 2-heptanoylpyridine are noteworthy because nitrogen-containing aromatic heterocyclic compounds are relatively rare in mammalian exocrine secretions. Pyridine and pyrazine derivatives have been found in male rabbit fecal pellets (Goodrich et al., 1981) and muscopyridine in the secretion of the scent gland of the musk deer (Biemann et al., 1957). Dimethylsulfone also has been found in the dorsal secretion of the springbok (Burger et al., 1981) and in the preorbital secretion of the suni, *Neotragus moschatus* (Burger et al., unpublished results). Finally, cholesterol and desmosterol are present in the secretions in small quantities (retention times beyond range shown in Figure 1).

The possibility exists that the carboxylic acids could be artifacts formed by autoxidation of aldehydes. Although this is a valid argument, and autoxidation could possibly make some contribution to the production of carboxylic acids, there does not seem to be a definite correlation between the quantities of the aldehydes and carboxylic acids present in the secretion. It is, for example, very unlikely that hexanal and heptanal could be oxidized to the corresponding acids whereas octanal is not.

Although a further 76 constituents were identified in the present study, many compounds, including several terpenoids, remained unidentified, mostly because of their uninformative and/or impure mass spectra. Comprehensive two-dimensional gas chromatography, preparative gas chromatography in conjunction with NMR analysis, and techniques such as HPLC-GC will have to be used in future projects to obtain further structural information on these constituents.

On principle, experimental animals were not sacrificed to obtain material for the present study and it was, therefore, not possible to collect material at regular intervals in order to determine whether the composition of the secretions is influenced by seasonal changes or other factors. Nevertheless, a relatively large number of samples of animals of different ages and from different regions of the country were analyzed over many years. Although the research was concentrated mainly on the qualitative composition of the secretions, very similar gas chromatographic profiles were obtained throughout. Some quantitative data on the major constituents present in the secretions of randomly selected individual male and female bontebok and blesbok in quantities higher than 1 ng/animal are given in Table 1. It must be pointed out that it is not possible to remove secretion quantitatively from the interdigital pouch, and the results are therefore based only on the fraction collected from the interdigital pouch. The secretion from the male bontebok referred to in Table 1 was observed to have a lower than normal viscosity and the higher values obtained for this animal can therefore probably be attributed to more efficient removal of the secretion from the interdigital cavity. Secretions from some of the older animals were more viscous than those of young ones to the extent that they were quite difficult to remove from the interdigital pouch in some cases.

This difference appeared to be due to the absence of low-viscosity oils or waxes in older animals. Since this waxy material probably acts as a controlled-release substance, the secretions of the older animals are released from the secretion at a higher rate in the absence of the waxy material and therefore appear to have a stronger, more pungent smell to the human nose than those of the younger animals. At this stage it is not clear whether this difference in viscosity has any semiochemical significance or whether it is merely a consequence of the slower production of secretion in older animals.

The possibility was investigated that some of the constituents of the secretions may be produced by microorganisms in the interdigital cavity and are not secreted by the animal itself. A GC-MS analysis of an extract of the glandular tissue from the interdigital pouch (Figure 2) revealed the presence of very small quantities of some of the major constituents of the secretion. This is a somewhat inconclusive result as the presence of components of the secretion in the glandular tissue could be ascribed either to the impregnation of the tissue with compounds produced by microorganisms, production of the compounds by the gland, or to production of some of the compounds by the gland and the others by microorganisms. Comparison of the ratios in which the major compounds are present in the secretion and in extracts of the glandular tissue shows that the carboxylic acids are present in much higher concentrations in the glandular tissue than in the secretion. Although this result is again not conclusive, it was considered to be an indication that at least some of the compounds could be produced by microorganisms.

Two aerobic bacteria, *Bacillus brevis* (Dubos and Cattaneo, 1939) and *Planococcus citreus* (Sneath et al., 1986), were found to be present in the interdigital secretions of male and female animals from different populations of both subspecies. The absence of any other bacteria in the interdigital cavity can possibly be attributed to the production of the antibacterial agents gramicidine and tyrocidine by *B. brevis.* (Dubos and Cattaneo, 1939). The production of a yellow water-soluble pigment by *P. citreus* (Sneath et al., 1986) may explain the yellow coloration of the lower parts of the white hair surrounding the interdigital cavity.

The two bacterial species were grown on modified Hutner medium, and the metabolites produced by the bacteria analyzed in the normal manner. Complete qualitative analysis of the bacterial products lies outside the scope of the present research, but preliminary results indicate that the long-chain carboxylic acids, isopropyl hexadecanoate, and some of the ketones are produced in vitro by *B. brevis.* (*Z*)-6-Dodecen-4-olide is one of the major compounds produced by *P. citreus.* It is therefore not unlikely that some of the other constituents of the interdigital secretions of the bontebok and blesbok could also be produced by mututal metabolism by the bacteria of each other's metabolites. This possibility will be investigated in further work on the interdigital secretions of the bontebok and blesbok.

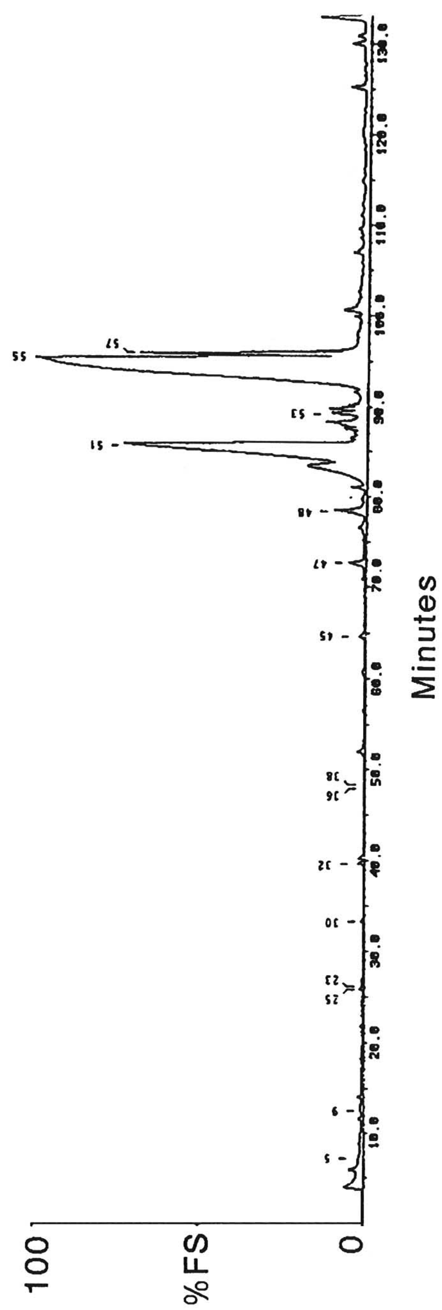


FIG. 2. Total ion chromatogram of an extract of interdigital glandular tissue from a male bontebok. Gas chromatographic conditions as in Figure 1.

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MAMMALIAN EXOCRINE SECRETIONS. XIII: CONSTITUENTS OF PREORBITAL SECRETIONS OF BONTEBOK, *Damaliscus dorcas dorcas*, AND BLESBOK, *D. d. phillipsi*

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Abstract—The 42 compounds identified in the pale yellow preorbital secretions of the bontebok, *Damaliscus dorcas dorcas*, and the blesbok, *D. d. phillipsi*, include acetic acid, 2-methylbutanoic acid, (*E*)-2-methyl-2-butenoic acid, benzoic acid, 4-hydroxybenzoic acid, saturated and unsaturated long-chain carboxylic acids, 4-hydroxybenzaldehyde, saturated and unsaturated, unbranched, long-chain alcohols and aldehydes, 2-heptanone, pentane, heptane, pepiritone, dimethylsulfone, isopropyl tetradecanoate, isopropyl hexadecanoate, hexadecyl acetate, δ-lactones, cholesterol, α-tocopherol, and squalene. Some qualitative and quantitative differences were found between the secretions of males and females of both subspecies.

Key Words—*Damaliscus dorcas*, mammalian semiochemicals, mammalian pheromones, exocrine secretions, preorbital secretions, chemical communication, mass spectrometry.

INTRODUCTION

The chemical composition of the preorbital secretions of several small South African antelope species has been studied in great detail in the Laboratory for Ecological Chemistry at the University of Stellenbosch. The results obtained

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so far indicate that the antelopes can be classified into three groups. In the first group—comprising grysbok, *Raphicerus melanotis*, (Burger et al., 1996) steenbok, *R. campestris*, (Burger et al. unpublished results), and oribi, *Ourebia ourebi* (Mo et al., 1995)—preorbital secretions consist largely of unbranched long-chain, saturated and unsaturated alcohols and some of their formates, as well as acetates, aldehydes, and carboxylic acids. Although some of these compounds are present in the secretions of two or all three of the species, there are marked qualitative and quantitative differences between the secretions. Long-chain formates and acetates are, for example, produced by the preorbital glands of both *Raphicerus* species, whereas only formates are present in the preorbital secretion of the oribi. As far as the position of the double bonds in the unsaturated constituents of the secretions and their stereochemistry are concerned, quite a large percentage of the unsaturated compounds are present in the secretions of both *O. ourebi* and *R. melanotis*. The relatively small number of unsaturated compounds present in the secretion of *R. campestris* have, in general, shorter chain lengths and double bonds located nearer to the functional groups of the compounds than those present in the other two species.

The structure of the preorbital glands of these animals differs from that of the second group of antelopes comprising grey duiker (*Sylvicapra grimmia*), red duiker (*Cephalophus natalensis*), and blue duiker, [*Philantomba monticola* (formerly *Cephalophus monticola*)]. Although the secretions of the grey duiker (Burger et al., 1990) and red duiker also contain long-chain compounds (Burger et al., unpublished results), the chain lengths tend to be generally shorter than those found in the first group. In addition to the ubiquitous unsaturated C_{18} carboxylic acids, the secretions of these two duikers contain one and two unsaturated compounds, respectively. Both contain γ-lactones and thiazoles (Burger et al., 1988), but only the red duiker secretion contains a relatively large number of spiroacetals (Burger et al., unpublished results). The blue duiker's preorbital secretion differs from those of the other two duikers in that it contains mostly short-chain compounds. It also contains several branched-chain compounds as well as a few unsaturated and aromatic compounds (Burger and Pretorius, 1987).

Probably the most conspicuous difference between the constituents of the blue duiker's preorbital secretion and the other secretions discussed so far is that the largest proportion of the blue duiker's compounds are secondary alcohols and ketones, whereas the constituents of the other secretions are mostly primary alcohols and their esters and the corresponding aldehydes and carboxylic acids. The preorbital secretion of the klipspringer, *Oreotragus oreotragus*, differs from those of the species mentioned above in that it contains only eight short-chain ketones and esters (Burger et al., 1997). As far as the complexity of the secretion and the average volatility of the organic constituents of its preorbital secretion are concerned, the klipspringer can be placed on its own in a third group.

Although general trends, such as the correlation between the rate at which

a secretion is produced and its complexity, are beginning to emerge, it became clear that antelopes from other tribes would have to be included in the study of the qualitative composition of the preorbital secretions of antelopes.

Inclusion of the two *Damaliscus* subspecies bontebok (*D. d. dorcas*) and blesbok (*D. d. phillipsi*) in the study was partly opportunistic because material was available. However, they also serve as examples of another taxonomic group of Bovidae and of antelope with a type of social organization very different from that of the species previously investigated. Grysbok, steenbok, oribi, and the duikers are solitary or occur in small groups (oribi) and occupy territories in which they live and on which they depend for food and other resources. Territorial advertising, which includes scent marking, is an ongoing process necessary for continued access to resources. Blesbok and bontebok are herd animals, in which the main function of territoriality is to provide dominant males with mating opportunities. Only that segment of the population is involved in active territorial advertising and defense and, since breeding is seasonal, it is limited in time. Such marked behavioral differences might be expected to be reflected in differences in the chemical composition of gland secretions.

The biology of bontebok and blesbok has been outlined in Burger et al. (1999). Prominent bulbous preorbital glands are present in both subspecies and are larger in males than in females (David, 1974; Lynch, 1974). In territorial male blesbok, secretion from the small round orifice of the gland accumulates on the face and stains the white face blaze yellowish black (Lynch, 1974). David (1974) did not observe face-staining in bontebok, although the glands are functional. Territorial male bontebok and blesbok deposit preorbital secretion on grass culms, and transfer it to the bases of the horns in a procedure known as glandular weaving. This procedure is also part of the challenge ritual between territorial males. In addition, Lynch describes how blesbok lying on their dung patches within the territory perform face-wiping, alternately wiping the preorbital glands first on the dung patch and then on the neck and shoulder. Territorial males advertise their presence and status in several ways. Their mere presence is obvious in the open habitat occupied by both subspecies. By defecating on particular spots within the territory, prominent dung patches are formed. Lynch (1974) considered them to serve as indirect marks in the case of blesbok, although David (1974) did not attribute a marking function to them in bontebok because bachelors and other territorial males took no notice of dung patches. Novellie (1981) demonstrated that a male bontebok can distinguish between his own fecal pellets and a stranger's, as well as between the pellets of individual males he had not previously encountered. He concluded that a territorial dung patch could communicate its owner's identity to neighboring territorial males. A ritualized defecation posture used by territorial male blesbok is thought to have a visual marking function, but the posture appears to be less exaggerated in bontebok (Lynch, 1974; David, 1974).

Lynch considers that preorbital glands play by far the major role in demar-

cating the male blesbok's territory. In contrast, David believes that preorbital secretion plays no functional role in territorial marking in bontebok. He found glandular weaving to be performed on average only once a day at the height of the rut, and did not observe any behavioral pattern signifying recognition of such scent marks. However, in tests with a captive male bontebok, Bigalke et al. (1980) found preorbital secretion to elicit much more interest than that from interdigital glands. They suggest that visual displays and active territorial defense may be most important, but that preorbital scent marks may provide familiar features in the territory holder's environment (self-reassurance).

In view of the fact that the behavior patterns of blesbok and bontebok are so similar and the preorbital secretions are chemically virtually identical, it seems unlikely that the glands play fundamentally different roles in territorial marking. The apparent differences may be a function of the different conditions pertaining in the study areas of David (1974) and Lynch (1974). The bontebok population numbered about 260 at a density of 1 per 10 ha, with a total of only 25–30 territorial males. Most of them remained territorial and retained small harem herds of averaging three individuals throughout the year. Lynch worked with about 1000 blesbok at a density of 1 per 3 ha. Furthermore, regular burning of segments of the grassland habitat resulted in a higher effective density since the blesbok concentrated on the most recently burnt segments of the reserve. Territorial behavior was much more strongly developed and much more seasonally limited in the blesbok studied, presumably as a result of more intense competition for access to females. Preorbital marking may, therefore, play a more significant role under these circumstances.

The decision to expand the study of the preorbital secretions of antelopes to include the two *Damaliscus* subspecies was taken towards the end of the investigation of the interdigital secretions of the bontebok, *Damaliscus dorcas dorcas*, and the blesbok, *D. d. phillipsi* (Burger et al., 1999), when it was possible to obtain preorbital secretions from a few male and female animals of both subspecies. In this paper the results are reported of an exploratory investigation that was mainly aimed at the determination of the qualitative composition of the preorbital secretions of these two subspecies.

METHODS AND MATERIALS

All the general and analytical methods used in this study were described in detail in a previous paper in this series (Burger et al., 1999).

Sample Collection and Preparation

Preorbital secretions were collected from male and female bontebok captured in the Bontebok National Park, Swellendam, and from a few blesbok cap-

tured in the Mountain Zebra National Park, Cradock, during routine game capture operations. The secretions were wiped from the preorbital glands with precleaned surgical gauze squares (Burger et al., 1999) and dressing forceps. The material was stored at −30°C in glass vials with Teflon-lined screw caps until used for analysis.

Gas chromatographic analyses were initially done by introducing pieces of surgical gauze containing preorbital secretion into the glass injector liner of the gas chromatograph, inserting the liner into the injector at ca. 35°C and ballistically heating the injector to 200°C. The volatile compounds desorbed from the gauze were cryotrapped on the capillary column with solid CO_2. Although this method worked well when quantitative desorption could be achieved below 100°C or when glass wool was used instead of surgical gauze, reactions apparently took place between the gauze and some of the constituents of the secretion at higher temperatures. This leads to the formation of artifacts and this sample introduction technique was therefore not employed in the present investigation. Acceptable results were obtained with Soxhlet extraction, as well as by wetting the gauze with dichloromethane, sonication, and centrifugal separation of the liquid phase from the gauze (Burger et al., 1999). The latter method gave a filtrate that separated into two layers. The dichloromethane layer was removed from underneath the water layer with a 1-ml syringe. Only acetic acid and some of the other polar aromatic and short-chain compounds were detected in low concentrations in the water layer. This method was used for the comparison of the secretions of individual animals.

Reference Compounds

Some of the compounds identified in the interdigital secretions of the bontebok and blesbok were available from previous projects in this series, while others are commercially available from Merck (Darmstadt, Germany), Fluka (Buchs, Switzerland), Aldrich (Milwaukee, Wisconsin), Sigma (St. Louis, Missouri), and BASF (Ludwigshafen, Germany). Octadecan-5-olide was synthesized during the present investigation.

Octadecan-5-olide. The δ-lactone was synthesized according to the procedure of Giese et al. (1984). A solution of mercury(II) acetate (12.99 g, 41 mmol) in water (45 ml) was added to 1-pentadecene (8.76 g, 42 mmol) in tetrahydrofuran (105 ml). The reaction mixture was stirred for 1 hr and treated with a mixture of acrylonitrile (6.46 g, 122 mmol), NaOH solution (2 M, 210 ml), and $NaBH_4$ (3.09 g, 81.6 mmol). The reaction mixture was stirred for 30 min and the product, 5-hydroxyoctadecanenitrile extracted with dichloromethane. The nitrile was hydrolyzed with NaOH solution (2 M, 42 ml) and the unsaponified constituents extracted with ether. The resulting emulsion was centrifuged to separate the phases. The water layer, containing the

saponified material, was acidified with H_2SO_4 (50%) and the organic material isolated in the normal manner to give octadecan-5-olide **39** (5.5 g, 46%) containing 3.4% 2-pentadecanol, and 0.8% 1-pentadecene (GC). HR-MS: m/z M^+ = 282.256, calcd. for $C_{18}H_{34}O_2$ 282.256. ^1H NMR ($CDCl_3$): δ = 3.61 [1H, m, —O—**CH**—$(CH_2)_2$], 2.41 (2H, t, —O—CO—$\mathbf{CH_2}$-), 1.2–2.0 [28H, m, —$(\mathbf{CH_2})_{12}$-, —O—CO—CH_2—$(\mathbf{CH_2})_2$-], 0.88 (3H, t, CH_3-). ^{13}C NMR ($CDCl_3$): δ = 172.01 (s, C-1), 76.62 (d, C-5), 35.89 (t, C-2), 31.95 (t, C-16), 29.39–29.68 (9t, C-7–C-15), 27.84 (t, C-4), 24.97 (t, C-6), 22.72 (t, C-17), 18.55 (t, C-3), 14.15 (q, C-18).

RESULTS AND DISCUSSION

The compounds identified in the preorbital secretions from the two subspecies are listed in Table 1, together with mass spectral data and some preliminary quantitative data. Some volatile constituents of the secretions are present in very low concentrations and will probably evaporate quite rapidly from the secretion or from deposited material, resulting in secretion or territorial marks enriched in the higher molecular mass constituents. There was wide variation in which constituents are present in the secretions collected from individual members of the two subspecies.

In contrast to most of the preorbital secretions so far analyzed, the secretions of the two *Damaliscus dorcas* subspecies are relatively simple, with a relatively small number of constituents present in concentrations higher than 0.1% of the organic fraction of the secretion. Most of the volatile organic constituents of the secretions were readily identified with low-resolution electron impact mass spectral data and comparison of these data with spectra in NBS and Wiley libraries. The molecular masses of some of the constituents were confirmed by chemical ionization mass spectral data obtained in a GC-CI-MS analysis with methane as reactant gas. With the exception of hexadecan-5-olide and heptadecan-5-olide, the proposed structures were all authenticated by retention time comparison with synthetic material. The retention time increments observed for the three δ-lactones were in agreement with their unbranched structures, and the identification of the C_{16} and C_{17} δ-lactones as unbranched were therefore accepted as correct without retention-time comparison with synthetic material. Although constituent **28** was present in secretions collected from males and females from both subspecies, it elutes as a broad peak. This constituent appears to be a substituted phenol which unfortunately could not be identified. No quantitative data are given for acetic acid as it elutes as a very broad band from the GC column used. The preorbital secretions of these animals consist largely of water and mucoid material in which the organic compounds are present in minute concentrations. As the ratio of water to organic material depends on various factors, such as, for

TABLE 1. COMPOUNDS FOUND IN THE PREORBITAL SECRETIONS OF THE BONTEBOK AND BLESBOK

Compounds identified	Normalized relative concentration (%)[a] Bontebok Male	Bontebok Female	Blesbok Male	Blesbok Female	EI mass spectral data [m/z (%)]
Alkanes					
Pentadecane[b,c]					169(1), 155(3), 141(5), 127(5), 113(10), 99(10), 85(38), 71(62), 57(100), 43(80), 41(53)
Heptadecane[b,c]					127(7), 113(9), 99(12), 85(45), 71(75), 57(100), 43(78), 41(57)
Alcohols					
3-Pentanol[b,c]					88(1), 60(9), 59(100), 57(14), 41(56)
1-Pentanol[b,c]					70(45), 57(25), 55(69), 42(100), 41(70)
1-Octen-3-ol[b,c,f]	[e]	1.4	[e]	2.6	99(12), 85(8), 72(15), 57(100), 43(55)
1-Henicosanol[b,c,d]	[e]		[e]		182(1), 167(2), 154(3), 139(7), 125(18), 111(40), 97(84), 83(94), 69(83), 57(100), 55(99), 43(95), 41(90)
Aldehydes					
Hexanal[b,c,d]	4.9	50.5	46.9	100	82(16), 72(20), 67(13), 57(53), 56(98), 44(100), 43(70), 41(94)
Heptanal[b,c,d]	0.2		0.4	3	114(1), 96(12), 86(18), 81(25), 71(30), 70(90), 57(50), 55(60), 44(100), 43(99), 41(98)
(*E*)-2-Heptenal[b,c,d,f]	13.5	26.1	29.2	1.4	112(3), 97(9), 84(20), 83(73), 70(37), 69(36), 68(33), 55(81), 41(100)
(2*Z*,4*Z*)-2,4-Heptadienal[b,c,d,f]	3.0	6.1	9.5	2.6	110(15), 95(5), 81(100), 67(16), 55(20), 53(26), 41(17)
(*E*)-2-Octenal[b,c,f]	0.4	0.9	7.2	5.7	111(2), 108(2), 98(7), 97(9), 83(40), 82(20), 70(65), 69(35), 55(78), 41(100)
Nonanal[b,c]					114(7), 98(22), 95(19), 82(24), 81(22), 70(35), 69(28), 68(23), 57(100), 44(47), 43(70), 41(85)
Decanal[b,c]					122(5), 112(28), 110(22), 106(9), 96(27), 82(37), 70(52), 57(90), 55(75), 44(46), 43(90), 41(100)

TABLE 1. CONTINUED

Compounds identified	Normalized relative concentration (%)[a]				EI mass spectral data [m/z (%)]
	Bontebok		Blesbok		
	Male	Female	Male	Female	
(2*Z*,4*Z*)-2,4-Decadienal[b,c,d,f]	9.5	5.0	52.6	25.9	152(4), 123(4), 109(2), 95(12), 83(14), 81(100), 67(21), 55(20), 41(35)
(2*E*,4*E*)-2,4-Decadienal[b,c,f]	16.3	100	100	66.3	152(4), 123(2), 109(2), 95(9), 81(100), 67(18), 55(14), 41(27)
4-Hydroxybenzaldehyde[b,c]					122(88), 121(100), 107(4), 93(40), 74(8), 65(48), 51(10), 39(31)
Ketone					
2-Heptanone[b,c,d]	*e*			*e*	114(4), 99(3), 85(3), 71(17), 59(10), 58(62), 43(100)
Carboxylic acids					
Ethanoic acid[g,h]					
2-Methylbutanoic acid[b,c,i]	0.6		2.6		87(24), 74(100), 57(60), 41(50)
(*E*)-2-Methyl-2-butenoic acid[b,c,i]	100	40.0	43.1	11.4	100(96), 85(36), 82(24), 55(100), 54(40), 53(34)
Hexanoic acid[b,c,d]	0.6	0.2	8.3	3.1	87(14), 73(51), 60(100), 55(28), 41(37)
Benzoic acid[b,c,i]					122(90), 105(100), 77(76), 51(14), 50(40)
Dodecanoic acid[b,c,d]					143(6), 129(34), 115(18), 101(20), 87(19), 73(100), 60(96), 55(76), 43(85), 41(90)
4-Hydroxybenzoic acid[b,c,i]					138(100), 121(89), 93(48), 65(42), 53(10), 45(8)
Tetradecanoic acid[b,c,d]	0.6	38.0	0.3		228(9), 199(3), 185(22), 171(8), 157(4), 143(11), 129(40), 115(13), 101(10), 97(15), 85(25), 73(100), 60(80), 55(67), 43(70), 41(77)
Pentadecanoic acid[b,c,d]	1.3	28.1		2.6	242(6), 213(5), 199(15), 185(12), 171(8), 157(11), 143(17), 129(30), 115(12), 101(8), 97(15), 85(24), 73(100), 60(90), 55(77), 43(92), 41(81)
(9*Z*,12*Z*)-9,12-Hexadecadienoic acid[b,c]	13.7	20.0	19.3	7.3	252(5), 163(2), 149(4), 135(10), 121(11), 109(17), 95(45), 81(91), 67(100), 55(68), 41(65)

Hexadecanoic acid[b,c,d,f]	23.7	62.4	21.2	4.7	256(8), 227(4), 213(12), 199(5), 185(10), 171(8), 157(11), 143(17), 129(36), 115(12), 101(8), 97(15), 85(24), 73(100), 60(80), 57(73), 55(68), 43(78), 41(60)
Heptadecanoic acid[b,c,d]	2.1	5.2	0.3	1.6	270(10), 241(4), 227(13), 213(6), 199(4), 185(15), 171(14), 157(7), 143(9), 129(35), 115(15), 101(7), 97(25), 85(28), 73(100), 60(87), 57(82), 55(87), 43(97), 41(77)
(9Z,12Z)-9,12-Octadecadienoic acid[b,c,d]	36.8	30.5	66.3	7.5	280(5), 164(2), 150(4), 136(5), 123(9), 109(23), 95(55), 81(86), 67(100), 55(54), 41(44)
Octadecanoic acid[b,c,d,f]	21.5	31.5	22.0	5.2	284(12), 255(2), 241(11), 227(4), 213(3), 199(6), 185(16), 171(7), 157(3), 143(6), 129(36), 115(11), 101(8), 97(26), 85(32), 73(100), 60(78), 57(77), 55(75), 43(90), 41(68), 43(100)
Nonadecanoic acid[b,c]					298(6), 255(12), 241(5), 227(9), 213(7), 199(9), 185(9), 171(7) 157(3), 143(6), 129(32), 115(11), 101(8), 97(27), 85(42), 73(100), 60(70), 57(80), 55(100), 43(95), 41(85)
Lactones					
Hexadecan-5-olide[b]	0.3	[e]	0.2	[e]	236(3), 192(2), 165(2), 152(6), 134(5), 123(4) 114(13), 109(12), 99(100), 83(23), 71(48) 55(66), 43(49), 41(56)
Heptadecan-5-olide[b,f]	1.3	4.7	4.9	0.5	250(3), 206(6), 151(5), 134(5), 123(4), 114(18), 99(100), 83(33), 71(52), 55(72), 43(60), 41(48)
Octadecan-5-olide[b,c,f]	6.6	8.8	13.0	2.6	264(2), 220(5), 180(1), 166(1), 151(3), 134(4), 125(6), 114(15), 99(100), 83(28), 71(49), 55(58), 43(58), 41(45)
Esters					
Isopropyl tetradecanoate[b,c,f]					229(15), 228(19), 211(15), 199(1), 185(8), 171(5), 143(6), 129(15), 111(6), 102(38), 97(12), 85(16), 73(30), 60(58), 57(43), 55(43), 43(100), 41(55)

TABLE 1. CONTINUED

Compounds identified	Normalized relative concentration (%)[a]				EI mass spectral data [m/z (%)]
	Bontebok		Blesbok		
	Male	Female	Male	Female	
1-Hexadecyl acetate[b,c]	3.1	*e*	0.2	*e*	224(1), 196(3), 153(2), 139(3), 125(8), 111(20), 97(37), 83(50), 69(42), 61(34), 55(53), 43(100)
Isopropyl hexadecanoate[b,c,d]	*i*	*i*	*i*	*i*	257(14), 256(19), 239(12), 213(5), 199(2), 185(4), 171(4), 129(15), 111(7), 102(44), 97(15), 85(20), 73(34), 60(67), 57(55), 55(49), 43(100), 41(52)
Terpenoids					
Piperitone[b,c]		*e*		*e*	152(15), 137(22), 110(80), 109(30), 95(54), 91(13), 82(100), 65(16), 54(20), 41(34)
Squalene[b,c,d]	7.1	38.6	7.2	1.4	191(3), 175(2), 161(2), 149(5), 137(7), 123(7), 121(9), 109(7), 95(15), 93(10), 81(49), 69(100), 55(8), 41(24)

Steroid					
Cholesterol[b,c,d]	*i*	*i*	*i*	*i*	386(21), 368(17), 353(17), 326(4), 301(25), 275(33), 255(20), 231(14), 213(32), 199(14), 185(12), 173(18), 159(37), 145(60), 133(34), 119(42), 105(75), 95(67), 81(73), 67(52), 55(86), 43(100), 41(65)
Other					
Dimethylsulfone[b,c,d,i]	0.6	4.4	0.4	2.6	94(57), 79(100), 65(2), 64(4), 63(7), 48(7), 45(8)
α-Tocopherol[b,c]	*i*	*i*	*i*	*i*	430(30), 205(10), 165(100), 164(35), 149(3), 136(4), 121(6), 69(6), 57(14), 43(24)
Unidentified[i]	*e*	1.5	*e*	*e*	194(40), 175(7), 166(6), 148(18), 138(10), 133(14), 121(48), 120(38), 108(100), 107(98), 91(20), 77(48), 65(15), 53(10), 51(10), 45(13)

[a]Normalized relative concentrations. Concentrations lower than 0.1% are not given.
[b]Low-resolution EI-MS.
[c]Retention comparison with authentic synthetic material.
[d]Also present in the interdigital secretions of *D. d. dorcas* and *D. d. phillipsi.*
[e]Not detected.
[f]Low resolution $CI(CH_4)$-MS.
[g]Found in small concentrations in the water layer. Elutes as a broad band, barely distinguishable from the base line.
[h]Not determined.
[i]Broad peak.

example, atmospheric conditions and the rate at which individual animals produce the secretions, the quantitative results are not given as the mass of each compound present in a secretion, but as the percentage in which a compound is present in the organic fraction of the secretion.

The quantitative data given in Table 1 seem to indicate that piperitone, 1-octen-3-ol, henicosanol, 1-hexadecyl acetate, and hexadecan-5-olide could possibly play a role in sexual recognition since these compounds appear to be completely absent from the secretions of one of the sexes. However, these compounds, as well as the unidentified constituent **28**, were found to be present in varying concentrations in the secretions of other individuals of both sexes and subspecies. The variation in the quantitative composition of the secretions investigated in the present study could, however, be interpreted in terms of the preorbital secretion being employed for individual recognition. Even to the human nose, mixtures of chemicals composed according to the quantitative data in Table 1 will smell vastly differently. Ideally, the preorbital secretions of a larger sample of related as well as unrelated animals from different social levels will have to be analyzed to find out whether the preorbital secretion is indeed employed for individual recognition. Several of the constituents of the preorbital secretions that are not present in other semiochemical secretions of the antelopes, such as piperitone, (*E*)-2-heptenal, and (*E*)-2-methyl-2-butenoic acid, have a strong smell to the human nose and are, in this respect, different from the majority of long-chain compounds present in these secretions. These compounds could possibly be the most likely candidates for behavioral tests with synthetic materials.

Although 19 of the compounds present in the preorbital secretions also have been identified in the interdigital secretions of the two subspecies (Burger et al., 1999), no transfer of material between the two glands appears to take place. (*E*)-2-Methyl-2-butenoic acid, one of the major constituents of the preorbital secretions, for example, is not present in the interdigital secretions and only one ketone was found in trace quantities in some preorbital secretions, whereas the interdigital secretions contain several ketones in relatively large quantities.

Many of the compounds present in the preorbital secretions of the two subspecies have already been found in the exocrine secretions from other South African antelope species. The present study has added 12 compounds to the list and has brought the total number of different compounds from the semiochemical secretions of these animals to 350.

Acknowledgments—Support by the University of Stellenbosch and the Foundation for Research Development, Pretoria, of research reported in this paper is gratefully acknowledged. The authors are indebted to the National Parks Board for permission to collect secretions from bontebok and blesbok in the nature reserves mentioned in this publication.

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MAMMALIAN EXOCRINE SECRETIONS. XIV: CONSTITUENTS OF PREORBITAL SECRETION OF STEENBOK, *Raphicerus campestris*

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Abstract—In a study aimed primarily at qualitative comparison of the organic constituents of the preorbital secretion of the steenbok, *Raphicerus campestris*, with those previously found in the preorbital secretion of the grysbok, *R. melanotis*, 109 compounds were identified in the secretion of the steenbok. Although the secretions from the two antelope are similar in that they are mostly long-chain, unbranched, saturated and unsaturated alcohols and various derivatives of these alcohols, only 22 of the identified compounds are present in both secretions. This is a small percentage of the more than 260 compounds present in the secretion of the steenbok, which is much more complex than that of the grysbok.

Key Words —*Raphicerus campestris*, mammalian semiochemicals, mammalian pheromones, exocrine secretion, preorbital secretion, territorial marking.

INTRODUCTION

Following identification of 34 constituents of the preorbital secretion of the grysbok, *Raphicerus melanotis*, in an early study of the chemical basis of the territorial marking behavior of this antelope (Le Roux, 1980; Burger et al., 1981a), behavioral tests and electrophysiological experiments were done. The secretions of conspecific animals as well as individual synthetic compounds were used in an attempt to find out whether some of the constituents have specific semiochemical functions. These experiments did not supply clear answers to any of the ques-

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tions that arose from observation of the animals' territorial behavior and from the results of the chemical characterization of the secretion (Le Roux, 1980). It is still not clear whether the small quantitative differences between male and female secretions have any semiochemical significance, especially because the quantitative differences between male and female secretions are almost exclusively observed in the very high molecular mass range. Although small quantitative differences were observed between the secretions of individual females, these differences probably have no semiochemical significance as far as the territorial behavior of the grysbok is concerned, because territorial marking with preorbital secretion has not been observed in female grysbok. Since information on the composition of the preorbital secretion of a related species could possibly shed light on the function of the preorbital secretions of these animals in their territorial behavior, an exploratory investigation of the qualitative composition of the preorbital secretion of the steenbok, *Raphicerus campestris*, was undertaken.

The genus *Raphicerus* comprises three species, the two mentioned above, and *R. sharpei* (Walker, 1968). The colloquial name of the steenbok is derived from Afrikaans for brick or stone, referring to the animal's even, rufous–brown to rufous–fawn color. The steenbok occurs on the African continent in two discrete areas, one in East Africa and the other in the southern parts of Africa with extensions of distribution into Angola and Zambia (Skinner and Smithers, 1990). Steenbok inhabit open grasslands, which must nevertheless provide some cover in the form of stands of tall grass, scattered bushes, or shrub. They do not occur in forests or thick woodland. Steenbok lead solitary lives except when a female has a lamb or when she is in estrus and is attended by a male. They are generally diurnal, but have some nocturnal activity, especially in areas where they are subject to disturbance (Skinner and Smithers, 1990).

Steenbok establish well-defined territories that both sexes will defend against trespassers, with established resting places, latrines, and preferred feeding places. Defense of these territories takes the form of displays rather than actual combat, and when fighting ensues between adults, it is only half-hearted and does not end in serious damage to the combatants. They have preorbital glands that show as dark marks just in front of the eyes, pedal glands between the hooves on the front and back feet, and a throat gland, all of which are presumed to be used for territorial marking (Skinner and Smithers, 1990).

METHODS AND MATERIALS

The black viscous preorbital secretion was collected, the organic material extracted with dichloromethane, and the constituents identified by the procedures and instrumentation described in detail by Burger et al. (1996).

Reference Compounds. Some of the compounds present in the preorbital secretion are commercially available, while others were available from previous

research projects in this series. Certain compounds were synthesized during the present investigation according to published procedures.

Alkyl and alkenyl formates were synthesized by the uncatalyzed reaction of the respective long-chain alcohols with an excess of formic acid (Burger et al., 1996). The final products generally contained less than 1% (GC) of the unchanged alcohols.

Alkyl acetates were synthesized by the esterification of the appropriate long-chain alcohols with acetic anhydride in the presence of a catalytic quantity of perchloric acid (Ongley, 1973). The final products contained only traces (GC) of the unreacted starting materials.

Isopropyl hexadecanoate was prepared similarly by heating isopropyl alcohol (1.92 g, 32 mmol), hexadecanoic acid (10 g, 39 mmol), and perchloric acid (5 drops) at 100°C for 4 hr. The reaction mixture was cooled to room temperature, diluted with ether, and the resulting solution washed free of acid with water. Isolation of the reaction product gave pure (GC) isopropyl hexadecanoate (9.51 g, 99.74%). The product was not distilled.

Hydroxyesters were prepared by the Al_2O_3-catalyzed reaction of long-chain 1,2-epoxyalkanes with the appropriate carboxylic acids as described by Burger et al. (1999).

Cyclohexadecanone was prepared by two routes. In the first, cyclopentadecanone was used as starting material and the carbocyclic ring was expanded by one carbon atom (Taguchi et al., 1974a,b). The final product was obtained in an acceptable yield, but it contained the starting material (cyclopentadecanone) and cyclohexadecanone in a 1 : 1 ratio.

The second synthesis consisted of selective hydrogenation of the double bond of 8-cyclohexadecenone (Aldrich, Milwaukee, Wisconsin). A solution of 8-cyclohexadecenone (3.92 g, 17 mmol) in glacial acetic acid (50 ml) containing a catalytic quantity of Pt on activated charcoal (10%) was hydrogenated until the theoretical volume of hydrogen had been consumed. The reaction mixture was diluted with water, the catalyst filtered off, and the reaction product extracted from the filtrate with pentane (100 ml). Work-up procedures and Kugelrohr distillation gave cyclohexadecanone (3.66 g, 90.46%) containing 4.6% (GC) of impurities. ^{13}C: $\delta(CDCl_3)$ 212,27 (s, C-1), 42.04 (2C, t, C-2 and 16), 26.54-27.68 (13C, t, C-3 to 15).

RESULTS AND DISCUSSION

Male and female secretions were collected once a month for 13 months to find out whether seasonal qualitative and quantitative changes could be observed in the composition of the volatile organic fraction of their secretions. The secretions of the male and female were found to be qualitatively identical regardless of the reproductive state of the animals. The glands of both male and female

appeared to be slightly and possibly insignificantly more productive while the female was in estrus. Identified compounds are listed in Table 1, and a typical total ion chromatogram of an extract of the preorbital secretion of a male steenbok is shown in Figure 1.

The constituents of the preorbital secretion were tentatively identified by comparison of their mass spectra with those in NBS and Wiley spectra libraries and a library of the mass spectra of compounds previously identified in mammalian secretions.

Early in the investigation it became clear that the preorbital secretion of the steenbok was the most complex preorbital secretion so far analyzed by the Laboratory for Ecological Chemistry and that it would not be possible to identify all of the constituents by GC and GC-MS retention time comparisons. Identification of the alkanes, alkanols, alkanals, alkanoic acids, alkyl formates, and alkyl acetates presented no problems, as authentic synthetic samples of almost all of these compounds were available for retention time comparison. In previous studies (Mo et al., 1995; Burger et al., 1996) the position of double bonds in unsaturated constituents of relatively complex mixtures was determined by GC-MS analysis of the reaction products obtained by treating whole extracts of secretions with dimethyl disulfide (Buser et al., 1983; Vincenti et al., 1987). The preorbital secretion of the steenbok, however, contains such a large number of unsaturated and doubly unsaturated compounds, and the total ion chromatogram of the mixture of DMDS derivatives was so complex that this technique did not supply unequivocal information on the position of the double bonds in the unsaturated constituents. Retention time comparison with synthetic compounds was, therefore, the only means of identifying some of these unsaturated compounds.

The long-chain 2-alken-1-ols have typical mass spectra with the ion at m/z 57 as the base peak. The configuration of the double bond was established by retention time comparison with two of these (*E*)-2-alken-1-ols. With a similar approach, a number of (*Z*)-6-alken-1-ols, four (*E*)-2-alken-1-yl methanoates, and two (*Z*)-6-alken-1-yl methanoates were identified in the secretion. The alcohols are not necessarily accompanied by their formic and acetic acid esters. These esters, therefore, do not appear to have been formed by simple esterification of the corresponding alcohols in the secretion.

The cycloalkanones present in the secretion have relatively prominent molecular ions, a base peak at m/z 55 and an abundant rearrangement ion at m/z 58. Cyclohexadecanone was synthesized as representative of this group of cyclic ketones for MS and retention-time comparison. The identification of three of the seven monounsaturated carboxylic acids present in the secretion as (*Z*)-9-alkenoic acids was confirmed by comparison with authentic synthetic material. With reasonable certainty the other two homologs can be assumed to be (*Z*)-9-dodecenoic acid and (*Z*)-9-tridecenoic acid.

It was relatively easy to characterize several long-chain hydroxyesters in

TABLE 1. CONSTITUENTS OF PREORBITAL SECRETION OF STEENBOK

No. in Figure 1	Compound	Remarks
2	Octane	*a,b*
8	Nonane	*a,b*
10	Decane	*a,b*
16	Dodecane	*a,b*
6	5-Methyl-3-hexanol	*a,b*
12	1-Octanol	*a,b*
14	1-Nonanol	*a,b*
18	1-Decanol	*a,b*
23	1-Undecanol	*a,b,d*
29	1-Dodecanol	*a,b,d*
35	1-Tridecanol	*a,b,d*
42	1-Tetradecanol	*a,b,d*
50	1-Pentadecanol	*a,b,d*
52	1-Hexadecanol	*a,b*
70	1-Icosanol	*a,b*
85	1-Tricosanol	*a*
91	1-Tetracosanol	*a,b*
97	1-Pentacosanol	*a,b*
101	1-Hexacosanol	*a,b*
105	1-Heptacosanol	*a,b*
22	(*E*)-2-Undecen-1-ol	*a*
28	(*E*)-2-Dodecen-1-ol	*a,b*
34	(*E*)-2-Tridecen-1-ol	*a*
41	(*E*)-2-Tetradecen-1-ol	*a,b*
45	(*E*)-2-Pentadecen-1-ol	*a*
9	(*Z*)-6-Hepten-1-ol	*a,c*
17	(*Z*)-6-Decen-1-ol	*a,c*
21	(*Z*)-6-Undecen-1-ol	*a,c*
27	(*Z*)-6-Dodecen-1-ol	*a,b,d*
61	(6*Z*,9*Z*)-6,9-Heptadecadien-1-ol	*a,c*
1	Hexanal	*a,b*
7	Heptanal	*a,b*
13	Nonanal	*a,b*
19	(2*Z*,4*Z*)-2,4-Decadienal	*a,b*
20	(2*E*,4*E*)-2,4-Decadienal	*a,b*
53	Cyclohexadecanone	*a,b*
58	Cycloheptadecanone	*a*
62	Cyclooctadecanone	*a*
67	Cyclononadecanone	*a*
71	Cycloicosanone	*a*
77	Cyclohenicosanone	*a*
3	Butanoic acid	*a,b*
4	3-Methylbutanoic acid	*a,b*
5	2-Methylbutanoic acid	*a,b*
15	Octanoic acid	*a,b*

TABLE 1. CONTINUED

No. in Figure 1	Compound	Remarks
24	Decanoic acid	*a,b*
36	Dodecanoic acid	*a,b*
47	Tetradecanoic acid	*a,b,d*
51	Pentadecanoic acid	*a,b,d*
57	Hexadecanoic acid	*a,b,d*
66	Octadecanoic acid	*a,b,d*
75	Icosanoic acid	*a,b,d*
33	(*Z*)-9-Dodecenoic acid	*a,c*
40	(*Z*)-9-Tridecenoic acid	*a,c*
46	(*Z*)-9-Tetradecenoic acid	*a,b*
56	(*Z*)-9-Hexadecenoic acid	*a,b*
64	(*Z*)-9-Octadecenoic acid	*a,b*
63	(9Z,12*Z*)-9,12-Octadecadienoic acid	*a,b*
26	1-Undecyl formate	*a,b,d*
32	1-Dodecyl formate	*a,b,d*
38	1-Tridecyl formate	*a,b,d*
72	1-Icosyl formate	*a,b,d*
78	1-Henicosyl formate	*a,b,d*
81	1-Docosyl formate	*a,b,d*
87	1-Tricosyl formate	*a,d*
93	1-Tetracosyl formate	*a,b,d*
99	1-Pentacosyl formate	*a,b,d*
103	1-Hexacosyl formate	*a,b*
106	1-Heptacosyl formate	*a,b*
110	1-Octacosyl formate	*a,b*
25	(*E*)-2-Undecen-1-yl formate	*a,c*
31	(*E*)-2-Dodecen-1-yl formate	*a,b*
37	(*E*)-2-Tridecen-1-yl formate	*a,c*
48	(*E*)-2-Pentadecen-1-yl formate	*a,c*
30	(*Z*)-6-Dodecen-1-yl formate	*a,b*
39	(*Z*)-6-Tetradecen-1-yl formate	*a,c*
44	1-Tridecyl acetate	*a,b*
55	1-Pentadecyl acetate	*a,b*
59	1-Hexadecyl acetate	*a,b*
43	Unidentified tridecen-1-yl acetate	*a*
54	Unidentified pentadecen-1-yl acetate	*a*
68	Unidentified nonadecen-1-yl acetate	*a*
79	Unidentified henicosen-1-yl acetate	*a*
86	Unidentified tricosen-1-yl acetate	*a*
88	Unidentified tetracosen-1-yl acetate	*a*
49	Isopropyl tetradecanoate	*a,b*
60	Isopropyl hexadecanoate	*a,b*
69	2-Hydroxyheptadec-1-yl acetate	*a,b*
74	1-Hydroxyoctadec-2-yl acetate	*a*
76	2-Hydroxyoctadec-1-yl acetate	*a,d*

TABLE 1. CONTINUED

No. in Figure 1	Compound	Remarks
83	1-Hydroxyicos-2-yl acetate	a,b
84	2-Hydroxyicos-1-yl acetate	a,b,d
89	1-Hydroxyhenicos-2-yl acetate	a
90	2-Hydroxyhenicos-1-yl acetate	a
95	1-Hydroxydocos-2-yl acetate	a
96	2-Hydroxydocos-1-yl acetate	a
100	2-Hydroxytricos-1-yl acetate	a
65	2-Hydroxytetradec-1-yl butyrate	a,b
73	2-Hydroxyhexadec-1-yl butyrate	a
80	1-Hydroxyoctadec-2-yl butyrate	a,b
82	2-Hydroxyoctadec-1-yl butyrate	a,b
92	1-Hydroxyicos-2-yl butyrate	a,b
94	2-Hydroxyicos-1-yl butyrate	a,b
102	1-Hydroxydocos-2-yl butyrate	a
104	2-Hydroxydocos-1-yl butyrate	a
11	Limonene	a,b
98	Squalene	a,b
107	Cholesterol	a,b
108	α-Tocopherol	a,b
109	Unidentified steroid	a

[a]Low-resolution mass spectrum.
[b]Retention time comparison.
[c]Position and configuration of double bonds, although not determined, are possibly as given.
[d]Also present in preorbital secretion of *Raphicerus melanotis*.

the secretion. The interpretation of the mass spectra of this compound type has been discussed by Burger et al. (1981b) and Le Roux (1980). The unbranched structures of the hydroxyesters were established by coinjection of the secretion and a number of authentic synthetic samples of representative compounds and by taking the retention time increments expected for the unbranched structures into consideration. It is possible that the hydroxyesters are formed by nucleophilic ring opening of long-chain oxiranes by ethanoic acid and butanoic acid. However, the difference in the ratios in which the 1-hydroxyalk-2-yl esters and 2-hydroxyalk-1-yl esters are formed cannot be explained in terms of such a route. 2-Hydroxyoctadec-1-yl and 1-hydroxyoctadec-2-yl butanoate are, for example, present in the secretion in a 1 : 1 ratio, whereas the 2-hydroxyicos-1-yl and 1-hydroxyicos-2-yl acetates are present in a ratio of 1 : 4. The stereochemistry of the hydroxyesters in this and several other mammalian exocrine secretions needs to be investigated further. The constituents of the secretion identified during this study are listed in Table 1.

In contrast to the preorbital secretion of the grysbok, *R. melanotis*, which has been almost fully characterized (Burger et al., 1996), the secretion of the

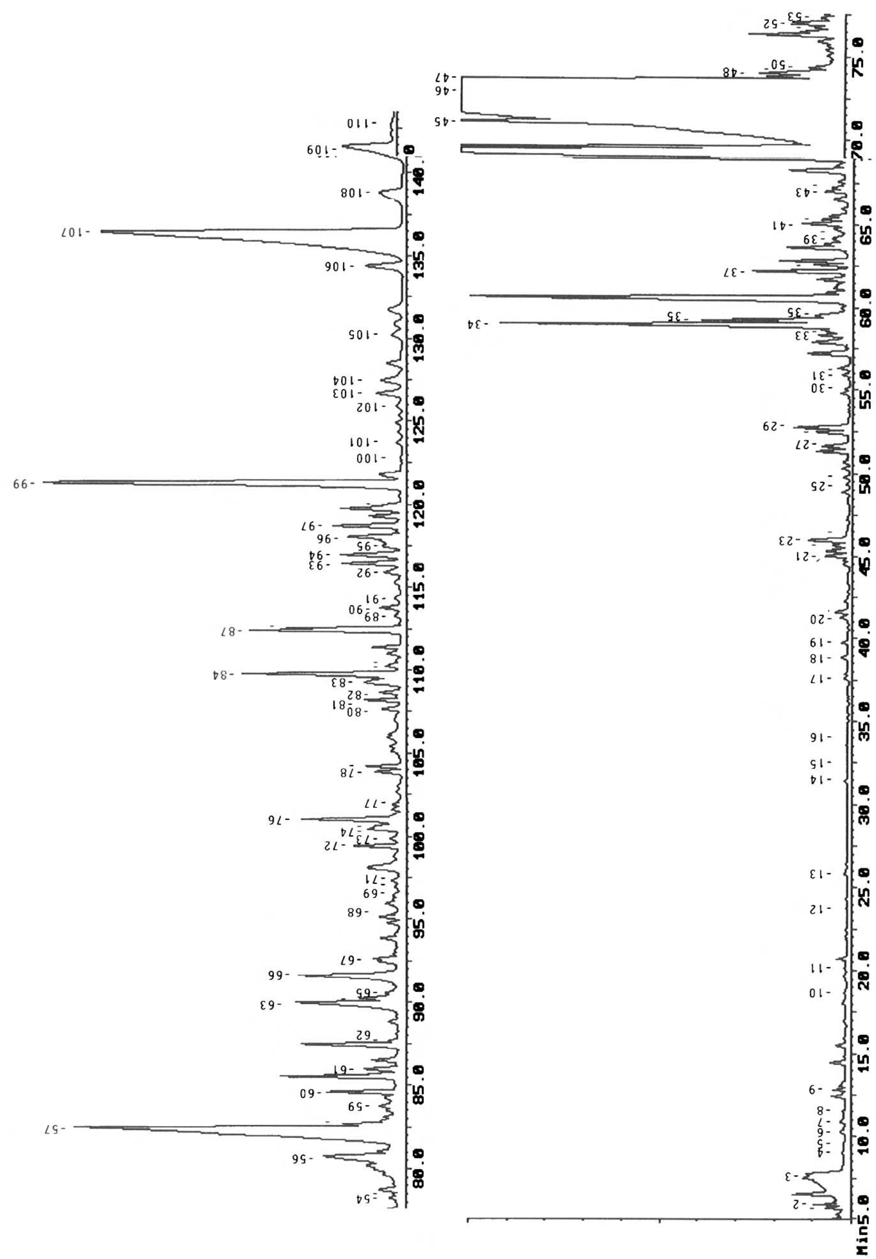

FIG. 1. Total ion chromatogram of an extract of the preorbital secretion of the steenbok. Glass capillary column coated with a 0.25-μm film of the apolar stationary phase PS-089-OH (95% dimethyl–5% diphenylsiloxane copolymer), programmed at 2°C/min from 40°C to 260°C (hold).

steenbok is much more complex. More than about 50% of an estimated 260 constituents remained unidentified in this study. This is largely due to the uninformative mass spectra of the long-chain compounds present in the secretion and the small concentrations in which they are present, which makes it impossible to isolate them for NMR and/or the determination of double bond positions. It is, however, clear that the unidentified compounds are of a similar long-chain unbranched type as those present in the grysbok and the steenbok secretions. Although the two secretions contain similar compounds, only 22 constituents are common to both secretions.

It is clearly not feasible to do field tests with individual constituents of the two secretions. However, the grysbok secretions contain certain lactones that are not present in the steenbok secretion. The steenbok secretion, on the other hand, contains a number of cyclic ketones not present in the grysbok secretion. There are also other differences as far as compound type is concerned. As a first approach to the semiochemical evaluation of the results presented here, field tests are planned in which the reaction of steenbok to their own secretion spiked with, for example, the lactones found in the grysbok secretion will be studied.

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COMPARATIVE METABOLISM OF DIETARY TERPENE, *p*-CYMENE, IN GENERALIST AND SPECIALIST FOLIVOROUS MARSUPIALS

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Abstract—The urinary metabolites of the monoterpene, *p*-cymene, were studied in three marsupial species: a generalist herbivore, the brushtail possum (*Trichosurus vulpecula*), and two specialist folivores, the greater glider (*Petauroides volans*) and the ringtail possum (*Pseudocheirus peregrinus*), as well as in the laboratory rat (*Rattus norvegicus*). Each animal was dosed, intragastrically, with single doses of *p*-cymene (0.37 mmol/kg and/or 1.49 mmol/kg). Urine and feces were collected for two 24-hr periods. Quantitative analysis of urinary metabolites by extraction, gas chromatography, and mass spectrometry gave a mean recovery of 64% (range 52–74%) of the administered dose in 48 hr for the four species. No fecal metabolites were found. A species-specific pattern of metabolite excretion was evident and reflected the natural occurrence of *p*-cymene (and terpenes in general) in the diet. If the metabolites excreted are grouped according to the total number of oxygen atoms added (one to four), then the rat excreted metabolites encompassing all degrees of oxidation, but predominantly a monooxygenated metabolite. The brushtail possum excreted metabolites having two, three, and four oxygen atoms added. The ringtail possum and greater glider only excreted metabolites with three or four oxygen atoms. Conjugation played a significant role in the excretion of oxidized metabolites in only the brushtail possums and the rat. These findings indicate that species encountering terpenes, such as *p*-cymene, in their normal diet have developed efficient oxidation pathways to eliminate them. This oxidative efficiency could also reduce the necessity

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for subsequent conjugation of metabolites which minimizes further demands on a nutritionally limited diet.

Key Words—*Trichosurus vulpecula, Pseudocheirus peregrinus, Petauroides volans, p*-cymene, metabolism, detoxification, *Eucalyptus*, terpene.

INTRODUCTION

One of the fundamental issues in animal–plant interactions is the detoxification of ingested plant secondary metabolites (PSMs). It has been 25 years since Freeland and Janzen (1974) hypothesized that the feeding behavior of herbivores is influenced by limitations of the body's mechanisms for detoxifying and excreting PSMs. The hypothesis was based on observational studies correlating dietary PSM concentrations with diet selection. Since that time we have learned little about the actual physiological processes and limitations of detoxification in wild animals.

The detoxification limitation hypothesis attempts to explain why browsing herbivores mostly consume varied diets (Freeland and Janzen, 1974; Freeland and Winter, 1975). A diet containing small amounts of a wide range of PSMs is argued to be less likely to saturate particular pathways of detoxification and excretion and so be preferable to a diet consisting of one plant type that would contain larger concentrations of fewer PSMs. Nonetheless, specialist herbivores have minimal dietary variation and must therefore have high-capacity detoxification pathways to deal with their PSM load. This study set out to test the hypothesis by comparing the metabolism of the PSM, *p*-cymene, in generalist and specialist herbivores.

The monoterpenes are a major group of PSMs occurring in many plants, including *Eucalyptus* spp. [up to 5% wet weight (Guenther, 1950; Boland and Brophy, 1991; Li et al., 1994)]. Despite the dominance of Australian forests by *Eucalyptus* spp., only four marsupials include eucalypt leaves in their diet, but these marsupials consume quantities of monoterpenes that would be toxic to other mammals (Whitman and Ghazizadeh, 1993).

In this study, we compared the metabolic fate of *p*-cymene, a common component of many *Eucalyptus* oils, in three of the marsupials and the laboratory rat. The three marsupial species studied cover a range of browsing herbivore niches. The brushtail possum (*Trichosurus vulpecula*) is a generalist herbivore (Kerle, 1984) feeding largely on leaves from a variety of plants, including eucalypts, but also supplementing its diet with insects, fruit, and flowers (MacLennan, 1984; Statham, 1984). The diet of the greater glider (*Petauroides volans*) consists exclusively of eucalypt leaves (Marples, 1973; Foley, 1987), making it a highly specialized folivore (Marples, 1973). The ringtail possum (*Pseudocheirus peregrinus*) consumes eucalypt leaves in most parts of its range but also eats leaves

and flowers of other species of canopy and understorey trees such as *Leptospermum* (Pahl, 1987). Ringtail possums, although essentially specialists, are not as reliant on eucalypt foliage as the greater glider. The rat (*Rattus norvegicus*) was included as a reference omnivore and to enable direct comparison with previous studies of the metabolism of *p*-cymene in this species (Walde et al., 1983).

p-Cymene (4-isopropyltoluene) has a relatively simple chemical structure (Figure 1). Metabolism in the rat, guinea pig, and rabbit has been reported (Ishida et al., 1981; Matsumoto et al., 1992; Walde et al., 1983). All these species exhibit a complex metabolic pattern with extensive oxidation of the isopropyl group, the methyl group, and ring.

METHODS AND MATERIALS

Materials. All reagents were obtained from commercial suppliers and were of analytical grade. *p*-Cymene was purchased from Aldrich Chemical Company, Inc. (Castle Hill, New South Wales, Australia).

Animals. The animals are described in Table 1. Hooded Wistar rats were obtained from stocks held in the University of Tasmania. Brushtail and ringtail possums were trapped or caught in *Eucalyptus* woodland near Hobart, Tasmania. Greater gliders were caught by hand in *Eucalyptus* forest near Townsville, Queensland. Approval for this study was obtained from the animal experimentation ethics committee of each institution. The marsupials were released at their site of capture at the end of the procedures.

All animals were kept in a light and temperature controlled room and housed individually in appropriate metabolism cages during the dosing experiments. The room was maintained at about 20°C with a 12-hr light–dark cycle.

It was desirable for each species to be maintained on a terpene-free diet during experiments. However, the greater glider is an obligate *Eucalyptus* leaf eater, and therefore it was not possible to feed them an artificial or terpene-free diet. Ringtail possums were transferred to an artificial diet with considerable difficulty.

Rats were fed standard pellets and brushtail possums a mixed diet of fruit and vegetable. The greater glider was maintained on a mixed diet of leaf from *E. crebra*, *E.* (=*Corymbia*) *citriodora*, *E.* (=*Corymbia*) *polycarpa*, and *E. tereticornis.* The day before dosing, gliders were offered only *E. polycarpa* foliage, because this leaf was found by analysis to contain <0.1% total terpenes with no *p*-cymene. This allowed a minimal washout period for the monoterpenes present in the mixed eucalypt diet. Urine collected immediately prior to dosing gliders confirmed no interference with *p*-cymene metabolites from residual dietary terpenes. The ringtail possums were weaned from a diet of *E. pulchella* to an artificial diet (mixed grated apples, bananas, wheat breakfast biscuits (Weetbix,

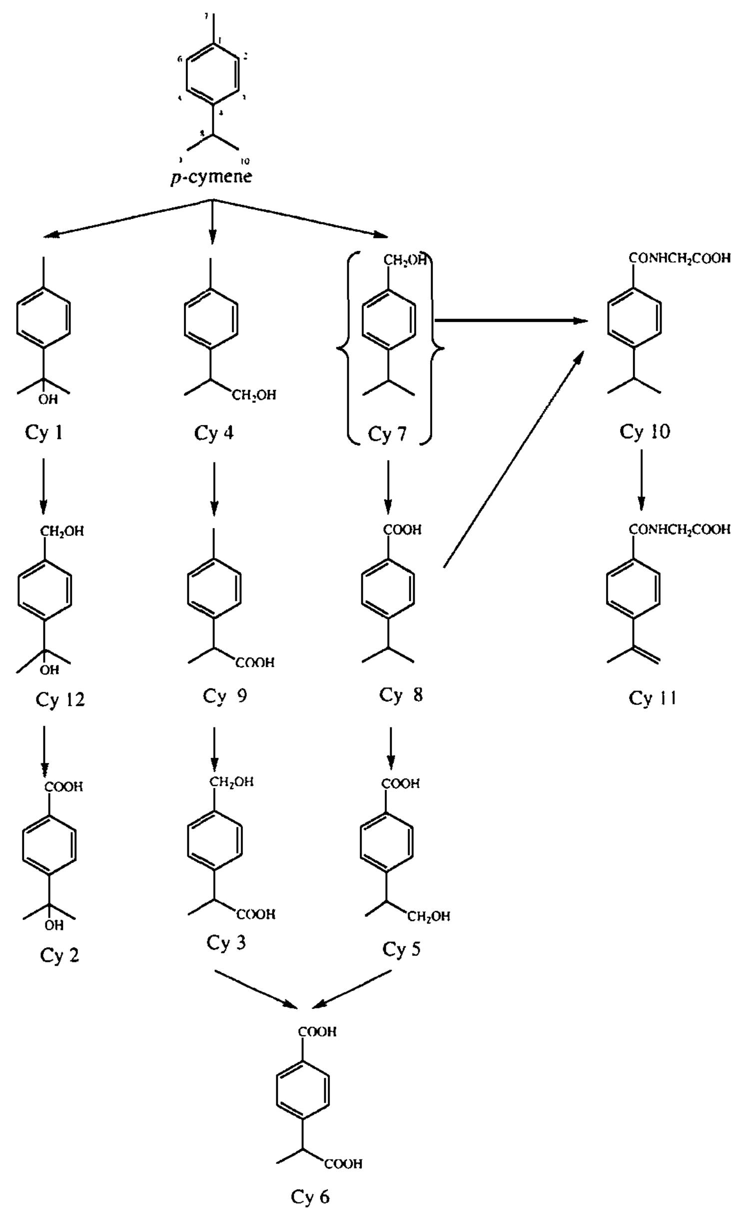

FIG. 1. Chemical structures of *p*-cymene metabolites and probable metabolic pathways in all four species. All metabolites identified are shown in their underivatized forms. Cy 7 was not found in this study.

TABLE 1. ANIMALS USED IN THE STUDY

Species	Common name	Females (*N*)	Males (*N*)	Weight (kg, mean ± SD)	Doses (mmol/kg)[a]
Marsupialia					
Phalangeroidea					
Trichosurus vulpecula	Brushtail possum	4	2	3.20 ± 0.90	0.37, 1.49
Petauroidea					
Petauroides volans	Greater glider	5	1	0.66 ± 0.07	1.49
Pseudocheirus peregrinus	Ringtail possum	1	2	1.00 ± 0.13	0.37
Eutheria					
Rodentia					
Rattus norvegicus	Rat	4	2	0.26 ± 0.05	0.37, 1.49

[a]Doses of *p*-cymene were administered per os.

Sanitarium Health Food Company, New South Wales, Australia), ground lucerne chaff, rice hulls, and casein) over a period of about three weeks.

Dosing and Urine Collection. Six animals from each species were used for dosing experiments, except the ringtail possum in which three animals were used. Animals were dosed by gavage. A curved, blunt syringe needle (18G × 5 cm), with a round bulb soldered to the tip, was used for rats. A flexible Gentle-Feed pediatric feeding tube (8.0F × 38 cm, Mallinckrodt Medical) was used for the marsupials.

On each occasion, animals received a single oral dose (0.37 mmol/kg or 1.49 mmol/kg) of *p*-cymene in peanut oil. These doses are, respectively, half and double the 100 mg/kg used previously in rats and guinea pigs (Walde et al., 1983) and were chosen to examine the dose-dependence of *p*-cymene metabolism. Because the species differed greatly in size, fresh *p*-cymene mixtures were prepared for each to provide appropriate dose volumes: rat, 0.06 and 0.30 mmol/ml; brushtail possum, 0.15 and 1.04 mmol/ml; greater glider, 1.12 mmol/ml, and ringtail possum 0.22 mmol/ml. Both doses (0.37 mmol/kg and 1.49 mmol/kg) were administered to the rats and brushtail possums. Only the higher dose was administered to the greater gliders due to time limitations, and the ringtail possums received only the lower dose to minimize stress on the animals as they did not seem to adapt well to captivity or the artificial diet.

Urine and feces were collected for 48 hr (two 24-hr periods) after the dose. Control samples were taken prior to dosing. Urine was collected into containers, placed in crushed ice, and then kept at −18°C until analyzed. Feces were separated from the urine by wire meshing placed over the urine collecting funnels, then stored at −18°C until analyzed. The volumes of urine (including funnel washings) and weights of feces were measured.

Analyses. Urine samples were analyzed to quantify the major total and free metabolites excreted by each species. To determine the total metabolites, urine samples were first hydrolyzed with β-glucuronidase plus aryl sulfatase as previously described (McLean et al., 1993).

Free metabolites were analyzed by placing a 1-ml sample of urine (or diluted urine) into a centrifuge tube along with an internal standard (125 μg 2,5-dimethylbenzoic acid in 50 μl methanol). The urine was acidified to pH 1 and then extracted into ethyl acetate and derivatized with diazomethane (McLean et al., 1993). The samples were then analyzed by gas chromatography.

Metabolites were identified by using a Hewlett-Packard (HP) 5890 gas chromatograph and HP 5970B mass-selective detector (GC-MSD) with HP 59970A Chemstation software (Hewlett-Packard Australia Ltd., Melbourne, Australia). The Chemstation software was modified to enable exporting of mass spectra to the NIST MS Search Program (NIST/EPA/NIH Mass Spec Library, Version 1.6, US Department of Commerce), for searching both the NIST and specific terpene libraries of mass spectra. Chromatography was carried out on a 25-m HP-1 capillary column (0.32 mm ID, coated with 0.52 μm cross-linked 1% phenyl methyl silicone). GC-MSD operating conditions were: splitless injector at 250°C; detector at 300°; oven from 60 to 190°C at 5°C/min, then from 190 to 290°C at 30°C/min and held at 290°C for 5 min; and carrier gas He at a pressure of 12 psi. MSD conditions required a solvent delay of 2 min, and a mass range of 40–450 was selected by using 1.5 scans/sec.

Mass spectra derived from *p*-cymene metabolites were compared by using probability-based comparisons to mass spectral data published by Ishida et al. (1981) and Walde et al. (1983) that had been entered into a custom made library with the NIST MS Search Program.

Quantitative analyses used a Varian 3300 gas chromatograph fitted with a Varian 1077 split/splitless capillary injector, flame ionization detector (FID) and Star Workstation (Version A.2, Varian Pty Ltd, Walnut Creek, California). Chromatography was carried out on a 25-m HP-5 capillary column (0.52 mm ID, coated with 0.25 μm cross-linked 5% phenyl methyl silicone). GC conditions were: injector at 250°C, split ratio 1 : 20; carrier He at 9 psi; oven from 120 to 190°C at 5°C/min, then from 190 to 290°C at 30°C/min, hold at 290°C for 2 min; and detector at 300°C.

Trimethylsilyl (TMS) derivatives were prepared by drying 20 μl ethyl acetate extract with a stream of N_2 in a heating block (40°C). Then, 20 μl *N,O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA, Alltech Associates (Aust) Pty. Ltd.) was added and the vial capped and heated at 60°C for 20 min before GC analysis. TMS-derivatized metabolites reported by Walde et al. (1983) were specifically searched for in urine extracts.

Isolation of Metabolites. Cumic acid (Cy 8) was obtained commercially (Aldrich Chemical Co., Castle Hill, New South Wales, Australia) and was 99%

pure. Metabolites Cy 1, 2, 3, 5, 6, and 9 (see Table 4 below for chemical names) were isolated from urine to allow quantitation. Pooled urine for each species was acidified to pH 1 and extracted into ethyl acetate. The extract was methylated with excess ethereal diazomethane.

Preparative thin-layer chromatography (pTLC) provided a preliminary clean up of metabolites. Glass plates were prepared by using powdered silica gel, 60 GF 254 (Keiselgel, Merck). Derivatized urine extract was applied to the plates that were developed with ethyl acetate/hexane (50 : 50).

Metabolite zones, visualized under UV, were scraped from the plates and redissolved in methanol. Either a second pTLC development or semipreparative high-performance liquid chromatography (pHPLC) was used to further purify the metabolites. A Shimadzu HPLC instrument was used, with a DGU-4A degasser, two LC 9A pumps, and a SPD-10A UV-VIS detector (Shimadzu Scientific Instruments Inc.) fitted with a 1-ml injection loop (Rheodyne Incorporated, Cotati, California). The column was a 25- × 100-mm cartridge (prep Nova-Pak HR C18 6 μm 60 Å) with a Guard-Pak Cartridge guard column (Waters Pty Ltd., Milford, Massachusetts). The column was radially compressed in a Waters PrepLC 25 mm module at <1500 psi. The mobile phase was methanol–water (45 : 55), flow rate 8 ml/min. Peaks were detected by UV absorbance (210 nm), collected, the solvent removed by rotary evaporation, and the residue dried under vacuum.

The identities of the isolated metabolites were confirmed by GC-MSD and retention time on GC-FID. Purity of isolated metabolites determined by GC was >90%, except for Cy 3, which was 85%. None of the impurities interfered with quantitative analyses. The weight of each metabolite was corrected for its percentage purity.

Calibration curves were prepared for each major metabolite. Standard amounts of metabolites in 50 μl of methanol were added to 0.5 ml blank urine, together with the internal standard. Since the metabolites had been derivatized to their methyl esters during isolation, an initial hydrolysis step was required to restore them to their excreted form as free acids. This was done by adding 0.5 ml 0.025 M NaOH to each solution and gently shaking in a waterbath at 30°C. The hydrolysis process was monitored over time by observing the disappearance of methylated metabolites by GC. After complete hydrolysis of the methyl esters (about 4 hr), the aqueous dilutions were extracted and remethylated, as described above.

Calibration curves were linear over the required concentration ranges for each metabolite. Same-day and within-day repeatabilities were determined. The coefficients of variation for low concentrations were <20% (range 7.2–19.4%, $N = 5$) and for higher concentrations were <15% (range 4.7–14.9%, $N = 5$).

Fecal Analysis. Feces were analyzed for *p*-cymene metabolites because of the possibility of biliary excretion, as has been found to be extensive for menthol,

another monoterpene (Yamaguchi et al., 1994). A sufficient amount of distilled water was added to the 24 hr feces collection to produce a slurry, and aliquots were analyzed for free and total metabolites in a method similar to that used for urinary metabolites.

Leaf Analysis. The terpene composition of *E. polycarpa* was determined by ethanolic extraction (Ammon et al., 1985) and analyzed by GC-MSD, as described above.

RESULTS

The structure of each metabolite identified is shown in Figure 1. Chemical names and references for published mass spectral data are provided in Table 2, along with the purity of those metabolites isolated from urine for quantification purposes. Mass spectral data obtained from *p*-cymene metabolites are reported in Table 3.

The fraction of the administered dose of *p*-cymene recovered in 48 hr is reported in Table 4. In most animals, the majority (>90%) of the recovered metabolites were excreted in the first 24 hr. However, recovery in the brushtail possums during the first 24 hr was variable, ranging from 0 to 81% (median

TABLE 2. *p*-CYMENE METABOLITES FOUND

Metabolites		Quantified (Y/N)	Purity (%)[a]	Reference
Cy 1	2-*p*-tolypropan-2-ol	Y	92.7	Ishida et al., 1981
Cy 2	2-*p*-carboxyphenylpropan-2-ol	Y	91.6	Ishida et al., 1981; Walde et al., 1983
Cy 3	2-*p*-(hydroxymethyl) phenylpropionic acid	N	85.0	Walde et al., 1983
Cy 4	*p*-cymen-9-ol	N	—	Ishida et al., 1981
Cy 5	2-*p*-carboxyphenylpropan-1-ol	Y	96.0	Walde et al., 1983
Cy 6	2-*p*-carboxyphenylpropionic acid	Y	95.0	Walde et al., 1983
Cy 8	*p*-isopropylbenzoic acid (cumic acid)	Y	99.0	Ishida et al., 1981; Walde et al., 1983
Cy 9	2-*p*-tolypropionic acid	Y	—	Ishida et al., 1981; Walde et al., 1983
Cy 10	*p*-isopropylbenzoylglycine (cuminuric acid)	N	—	Walde et al., 1983
Cy 11	*p*-isopropenylbenzoylglycine	N	—	Walde et al., 1983
Cy 12	2-*p*-(hydroxymethyl) phenylpropan-2-ol	N	—	Walde et al., 1983

[a]Purity was estimated by GC analysis. Cy 8 was purchased commercially and Cy 9 was quantified using the Cy 8 calibration curve.

TABLE 3. RETENTION TIME AND MASS SPECTRAL DATA FOR EACH METABOLITE QUANTIFIED

Metabolite	Retention time (min)[a]	Derivative[b]	Significant EI ions (m/z) and relative abundance (%)[c]															
Cy 1	3.55	—	150	136	135	132	119	117	115	91	89	77	67	65	63	51	44	43
			6	6	49	30	3	4	4	14	3	4	3	8	5	4	3	100
Cy 2	9.48	M	194	179	163	137	135	105	91	77	59	43						
			2	100	11	16	7	13	12	14	16	78						
Cy 3	10.51	M	194	136	135	117	115	107	105	103	91	79	77	44				
			26	11	100	9	5	12	40	10	15	22	20	12				
Cy 4	4.70	M	150	120	119	117	115	104	91	77	65	44						
			17	13	100	17	8	6	28	10	7	4						
Cy 5	11.71	M	194	163	149	131	117	105	91	77	59	51						
			14	100	12	22	11	29	33	27	25	10						
Cy 6	11.87	M	222	191	163	131	104	103	91	77	59	51						
			30	14	100	16	14	20	15	15	18	6						
Cy 8	6.35	M	178	163	147	131	119	117	104	103	91	78	77	59	51	50	41	
			48	100	40	25	58	13	17	27	59	15	32	29	20	10	20	
Cy 9	5.31	M	178	120	119	117	103	91	77									
			20	11	100	12	5	19	7									
Cy 10	16.54	M	235	147	131	104	103	91	77									
			19	100	6	7	7	12	9									
Cy 11	16.88	M	233	201	145	117	116	115	102	91								
			2	100	11	16	7	13	12	14	16	78						
Cy 12[d]		TMS	310	295	133	131	115											
			0	100	14	30	4											

[a]Retention times were measured by GC-FID. See text for conditions.
[b]Methyl ester (M), TMS ether (TMS), or no derivative (–).
[c]Relative abundances are given in the second line of each entry.
[d]Cy 12 was identified by GC/MS as its TMS derivative and was not analyzed by GC-FID.

TABLE 4. URINARY EXCRETION OF *p*-CYMENE METABOLITES IN EACH SPECIES

		Rat		Brushtail possum		Greater glider	Ringtail possum
		[0.37 mmol/kg] (N = 6)	[1.49 mmol/kg] (N = 6)	[0.37 mmol/kg] (N = 6)	[1.49 mmol/kg] (N = 6)	[1.49 mmol/kg] (N = 6)	[0.37 mmol/kg] (N = 3)
Fraction (mean ± SD) of dose recovered[a]		0.645 ± 0.123	0.518 ± 0.085	0.615 ± 0.169	0.601 ± 0.137	0.734 ± 0.074	0.735 ± 0.071
Metabolite	O_n[b]	Percent (mean ± SD) of total urinary metabolites excreted in 48 hr[c]					
Cy 1	1	39.2 ± 2.5	34.6[d] ± 3.6				
Cy 2	3	19.5 ± 2.7	23.3[d] ± 1.1	4.4 ± 2.3	8.0[d] ± 1.8	12.1 ± 2.1	36.3 ± 3.8
Cy 3	3	tr	tr				22.6 ± 2.5
Cy 4	1	tr	tr	tr	tr		
Cy 5	3	10.3 ± 2.6	9.7 ± 2.0	56.8 ± 5.4	59.0 ± 9.7	42.0 ± 2.5	tr
Cy 6	4	14.3 ± 2.8	13.7 ± 1.2	18.4 ± 3.8	6.6[d] ± 1.9	45.9 ± 4.1	41.0 ± 2.6
Cy 8	2	16.7 ± 2.5	18.6 ± 3.5	6.6 ± 5.8	16.9 ± 10.3		
Cy 9	2			13.8 ± 2.9	9.5[d] ± 2.8		
Cy 10	2	tr	tr	tr	tr		
Cy 11	2			tr	tr		
Cy 12	2	tr	tr				

[a]Fraction of administered dose recovered in 48 hr. $N = 6$ for all species except the ringtail possum ($N = 3$).
[b]O_n = number of oxygen atoms introduced by oxidation of *p*-cymene.
[c]tr = trace (detectable, but not quantified), blank = not detected.
[d]Comparison between doses, $P < 0.05$, *t* test, unpaired.

15%). This variability appeared to be due to no, or minimal, urine output in some animals and reflected the possum's food and/or fluid intake. One ringtail possum excreted only 45% of the recovered urinary metabolites in the first 24 hr, whereas the other two ringtails excreted the majority in this period.

Results from the two 24-hr postdose urine collections were combined for the total metabolite production. The overall recovery of the *p*-cymene dose in each species was consistent, although relatively low, ranging from 0.52 to 0.74 of the dose (Table 4). There was no difference between doses in recovery of dose.

Metabolites excreted are reported for each species as the percent of total recovered metabolites (Table 4). In the rat and brushtail possum (which were given two different doses), the relative amounts of each metabolite differed to a small extent between doses, but the overall patterns were similar after each dose. Significantly different metabolite excretion patterns were clearly evident among the four species studied. The rat and brushtail possum excreted five metabolites in quantifiable amounts, while the specialist herbivores, the ringtail possum and the greater glider, excreted only three.

Metabolites excreted by each species were grouped according to the total number of oxygen atoms (one to four) acquired. Metabolites having the same degree of oxidation have been combined and are presented in Figure 2. A progression is evident from the rat, which excreted metabolites with all four levels of oxygenation, through the brushtail possum, which only excreted metabolites

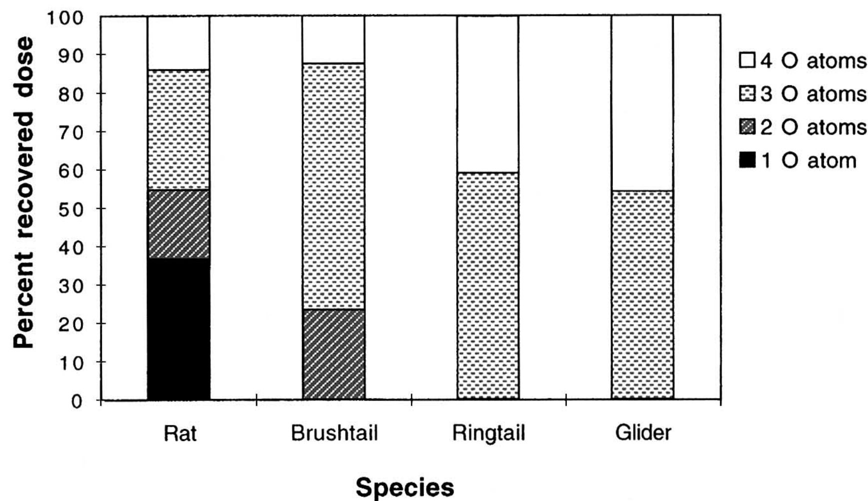

FIG. 2. *p*-Cymene metabolite excretion pattern for each species categorizing metabolites by number of oxygen atoms acquired.

TABLE 5. CONJUGATION OF METABOLITES EXCRETED IN RAT AND BRUSHTAIL POSSUM[a]

	Percent (mean ± SD) of total urinary metabolites			
	Rat		Brushtail	
Metabolite	Free	Conjugated	Free	Conjugated
Dose = 0.37 mmol/kg				
Cy 1	11.0 ± 4.5	28.9 ± 4.9		
Cy 2	10.5 ± 5.1	8.5 ± 2.6	4.4 ± 1.8	0.1 ± 1.3
Cy 5	5.9 ± 2.8	4.6 ± 3.2	28.1 ± 9.0	28.7 ± 12.3
Cy 6	6.9 ± 4.0	6.7 ± 4.6	13.2 ± 3.4	5.2 ± 1.5
Cy 8	0	16.7 ± 2.5	6.7 ± 6.5	0
Cy 9			7.5 ± 4.3	6.3 ± 3.6
Total	34.2 ± 12.5	65.8 ± 12.5	59.9 ± 18.2	40.1 ± 18.2
Dose = 1.49 mmol/kg				
Cy 1	30.4[b] ± 2.9	5.4[b] ± 2.3		
Cy 2	26.3[b] ± 2.0	0[b]	11.1[b] ± 6.7	0
Cy 5	11.0[b] ± 1.7	0[b]	23.8 ± 12.8	35.2 ± 16.8
Cy 6	12.0 ± 3.2	2.0 ± 1.4	7.7[b] ± 4.2	0[b]
Cy 8	2.3 ± 2.5	15.7 ± 4.4	2.8 ± 1.6	14.1[b] ± 9.4
Cy 9			4.0 ± 2.5	5.6 ± 3.2
Total	81.9[b] ± 6.5	18.1[b] ± 6.5	44.3 ± 21.0	55.7 ± 21.0

[a]The total values of urinary metabolites are calculated from the sum of individual animal metabolites and therefore are not always equal to the summation of values for each metabolite. Minor discrepancies between the results recorded in this table and Table 4 are due to the free and total metabolite concentrations being measured separately.
[b]Comparison between doses, $P < 0.05$ (t test, unpaired).

with two, three, or four oxygens, to the greater glider and ringtail possum which only excreted metabolites with three or four oxygens.

The percent of a metabolite excreted as a hydrolyzable conjugate was calculated by subtracting the percent of free metabolite (unhydrolyzed urine) from the percent of total metabolite (hydrolyzed urine). Conjugated metabolites were detected only in the rat and brushtail possum and are reported in Table 5. Neither the ringtail possum nor the greater glider excreted detectable amounts of hydrolyzable metabolites. Furthermore, glycine conjugated metabolites (Cy 10 and 11) also were not detected in the latter two species. Trace amounts of Cy 10 were detected in the rat and both Cy 10 and 11 in the brushtail possum.

The extent of conjugation of metabolites differed little between doses in the brushtail possum, while the rat excreted a considerably lower proportion of the urinary metabolites as conjugates after the higher dose (Table 5).

There was no unchanged *p*-cymene or *p*-cymene metabolites detected in the feces of any of the species.

DISCUSSION

Specialist and generalist marsupial herbivores had markedly different patterns of excretion of the terpene *p*-cymene. Three different patterns of metabolism were evident, providing a valuable insight into the mechanisms of detoxification employed by these folivores. First, the specialist herbivores excreted fewer metabolites of *p*-cymene compared to the generalists. Second, metabolites excreted by the specialists were all extensively oxidized (with the addition of at least three oxygen atoms), whereas the generalists excreted metabolites with a more varied range of oxidation (one to four added oxygen atoms). Finally, the role of secondary conjugation with glucuronic acid or glycine was of little importance in the specialist herbivores. We suggest that dietary limitations of specialist folivores favor the excretion of extensively oxidized metabolites of *p*-cymene, rather than glucuronide conjugates.

Number of Metabolites Excreted. In the rat, we identified five major and four minor metabolites. Walde et al. (1983) reported a total of 16 *p*-cymene metabolites in rat urine, nine of which were present in minor amounts (<4% or as trace amounts only). The five major metabolites identified in our study corresponded to the five predominant metabolites reported by Walde, although there were differences in relative abundances. Despite searching for key ions by using selective ion monitoring (SIM) by GC-MSD, seven of Walde's trace metabolites were not detected in this study. It may be that they were present below the detection limits of the assay and GC-MSD operating conditions used. The unidentified metabolites were four diols, a glycine conjugate, its immediate precursor, and a tertiary alcohol, cuminyl alcohol (Cy 7).

In the brushtail possum, we identified five major and three minor metabolites. In contrast, both specialist herbivores excreted only three major metabolites, while a trace of one minor metabolite (Cy 5) was detected in the ringtail possum.

Degree of Metabolite Oxidation. The greater glider and ringtail possum (i.e., the specialists) excreted only extensively oxidized metabolites (with three or more oxygen atoms added to the parent compound). In contrast, the rat excreted a range encompassing all degrees of oxidation, but predominantly the less oxidized metabolites. The metabolites excreted by brushtail possums tended towards the moderately oxidized metabolites (two to three oxygen atoms).

Importance of Metabolite Conjugation. Conjugation appeared to be more important in the elimination of *p*-cymene in the rat and brushtail possum than in the greater glider or ringtail possum. Glucuronidation is utilized by many animals due to its high capacity and its effectiveness in increasing the renal excretion of xenobiotics and endogenous waste compounds (Hirom et al., 1977; Caldwell, 1982). Brushtail possums have a low capacity to form sulfate conjugates [probably due to dietary deficiencies of sulfate (Roy, 1963)], and therefore, the gluc-

uronidation pathway becomes more important (Baudinette et al., 1980). Hinks and Bolliger (1956, 1957) also noticed that folivorous marsupials presented high levels of glucuronic acid or glucuronides in their urine compared to other marsupials. We would, therefore, except the hydrolyzable conjugates identified in this study to be glucuronides.

McLean et al. (1993) also found glucuronidation of terpenes, derived from dietary *E. radiata*, to be minimal in ringtail possums. When fed *E. radiata* for extended periods, the excretion of glucuronides increased over the first three days, but remained less (30%) than the total amount of oxidized terpene metabolites.

Conjugation is variable. The rat excreted a greater portion of metabolites as conjugates after the lower dose than after the higher dose, whereas the trend was in the opposite direction for the brushtail possum. The dose-dependent excretion in the rat indicates saturation of the glucuronidation pathway, which evidently has a higher capacity in the brushtail possum.

Conjugation of *p*-cymene metabolites with the amino acid glycine is also possible. The glycine conjugation pathway was active in all species studied, as the glycine conjugate of benzoic acid, hippuric acid, was excreted in each case. Walde et al. (1983) reported the following glycine conjugates of *p*-cymene in rats: 2% of administered dose was excreted as *p*-isopropylbenzoyl glycine (cuminuric acid, Cy 10) and a trace as *p*-isopropenylbenzoylglycine (Cy 11). We found only trace amounts of Cy 10 in the rat and of Cy 10 and 11 in the brushtail possum. McLean et al. (1993) found no glycine conjugates of the carboxylic acid metabolites of *E. radiata* terpenes in the ringtail possum. Interestingly, in the guinea pig, the major metabolite of *p*-cymene is the glycine conjugate, Cy 10 (Walde et al., 1983).

As mentioned, the marsupials studied cover the range of feeding niches from generalist browser (brushtail possum) to specialist herbivore (ringtail possum and the highly specialized greater glider). The different patterns of elimination of *p*-cymene in these animals may reflect their dietary classifications, which further reflects the nutritional quality of their respective diets. A diet consisting solely of eucalypt leaves is nutritionally limited, as it contains low levels of nitrogen compared to most other plants. It also contains large amounts of PSMs, resulting in a significant toxic challenge (Foley, 1987).

Herbivores would require adaptations to specialize successfully on a potentially toxic and nutritionally poor diet. An enhanced capacity of liver, and possibly other metabolically active tissues, to extensively oxidize compounds such as *p*-cymene and other terpenes would support this hypothesis. The similar extent of oxidation of metabolites excreted by the ringtail possum and greater glider, which not only share a similar herbivore niche, but are also closely related as members of the Marsupialia family *Pseudocheiridae* (Flannery, 1994), also adds weight to this hypothesis. Extensive oxidation of *p*-cymene enables the forma-

tion of highly polar metabolites that can be efficiently excreted without needing subsequent conjugation. This stratagem conserves the nutrients that would otherwise be required to form glucuronides and glycine conjugates.

The diet of the brushtail possum may be better nutritionally than that of the specialists, due to the wider variety of food consumed (Freeland and Winter, 1975). Greater variety would also result in the generalist herbivore being exposed to smaller amounts of a larger range of PSMs. A number of detoxification pathways with lower capacity would, therefore, be an advantage to the generalist herbivore, and our data showing a greater number of *p*-cymene metabolites in brushtail possums may support this. Freeland and Winter (1975) determined that brushtail possums are unable to maintain their body weight when fed a diet consisting solely of one species of eucalypt. They hypothesized that the cumulative dose of only a few PSMs exceeds the brushtail's detoxifying capacity and results in an inability to eat sufficient food to maintain body weight. Thus, it would seem the strategy employed by the brushtail possum requires a varied diet, in which the overall toxic load can potentially be great, but the detoxifying process is dispersed over numerous pathways. The results presented here show that this multiplicity of metabolic pathways can also occur with a single compound, *p*-cymene.

The rat would encounter low exposure to terpenoid compounds in its omnivorous diet. No refined mechanism for detoxifying these compounds would be expected to have developed. Significantly, the rat was the only species studied to excrete a monooxygenated metabolite of *p*-cymene, and this was its major metabolite.

The metabolic pathway of *p*-cymene shown in Figure 1 is based on the assumption that minor and trace metabolites are precursors of the major, more highly oxidized, metabolites. The absence of some precursors in the greater glider and ringtail possum suggests that the intermediate compounds are immediately further oxidized.

Others have examined the stereochemistry of terpene metabolism. Matsumoto et al. (1992) studied the metabolism of *p*-cymene in rabbits and found that the enzymatic oxidation of *p*-cymene occurred stereoselectively. Stereoselective metabolism has also been found in the metabolism of 1,8-cineole in brushtail possums (Carman and Klika, 1991, 1992; Matsumoto et al., 1992; Carman et al., 1994; Carman and Rayner, 1994). It would, therefore, be reasonable to expect that some of the metabolites excreted in the animals studied here would also show chiral characteristics, but this requires further investigation.

The regioselectiveness of sites of oxidation in the preferred metabolites has also received some attention in these works and in a review by Oguri et al. (1994). The cytochrome P-450 enzymes responsible for the oxidation of *p*-cymene seem to have different regioselective properties in the ringtail possum and the greater glider. Both species excreted Cy 2 and 6 as major metabolites,

but the ringtail excreted Cy 3 as an immediate precursor to Cy 6, while the greater glider excreted its structural isomer, Cy 5. This suggests that different enzymatic pathways are used.

In each species, a significant amount of the administered dose remains unaccounted for. We have a partial explanation. First, only major metabolites were quantified and, therefore, the contribution of minor metabolites has not been taken into account. This was more significant in the case of the rats and brushtail possums, which produced the greatest number of metabolites. Second, the possibility of excretion of unchanged *p*-cymene in urine, feces, or expired air should be considered. Unchanged *p*-cymene was not detected in the urine. Traces of *p*-cymene were found in feces of brushtail possums after oral administration by Southwell et al. (1980) but were not detected in this study. On the other hand, because of the volatile nature of *p*-cymene, it could be expected to be detected in expired air. Lee (1987) reported that a small fraction of *p*-cymene absorbed into the blood is excreted unchanged in expired air. Finally, urinary excretion was not followed after the second day, so any slowly excreted components of the dose would have been missed.

Conclusions made in a comparative study such as this are necessarily limited. An assumption is made concerning the equality of the "toxic challenge" administered to each species. A dose of 1.49 mmol/kg of *p*-cymene may be significantly more challenging for the rat than for the greater glider. Furthermore, the results observed from a single dose of one terpene may not accurately reflect what occurs in chronic dosing of mixed terpenes, as in a natural diet. Therefore, conclusions made are confined by the choice of the dosage regimen. Experience with chronic and/or larger dosing regimes are required to make more definite conclusions.

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ACTION OF EXTRACTS OF APIACEAE ON FEEDING BEHAVIOR AND NEUROPHYSIOLOGY OF THE FIELD SLUG *Deroceras reticulatum*

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Abstract—A systematic examination was made of the plant family Apiaceae (Umbelliferae) in which extracts of 33 species, representing 32 genera, were screened for antifeedant activity against the field slug *Deroceras reticulatum* by using an electrophysiological recording assay. In this assay, the olfactory sensory epithelium of the posterior tentacle of the slug was exposed to volatile components of the plant extracts presented in an airstream, and any subsequent activity of the olfactory nerve was recorded. Extracts of 22 species elicited a range of nervous activity in the preparation. A feeding bioassay was used to measure any change in consumption when extracts were added to a standard food. Statistical analysis of data obtained from both electrophysiological traces and the feeding bioassays identified extracts of *Petroselinum crispum*, *Conium maculatum*, and *Coriandrum sativum* as being the most neuroactive as well as the most antifeedant.

Key Words—*Deroceras reticulatum*, Apiaceae, neurophysiology, tentacle nerve preparation, antifeedant, bioassay.

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INTRODUCTION

Mollusks are capable of a high degree of selection in their feeding behavior. This is due to the presence of secondary metabolites contained within plants (Cates and Orians, 1975). These semiochemicals are detected by chemoreceptors located in the slug tentacles. In terrestrial mollusks, the posterior tentacles detect volatile airborne cues, while the anterior tentacles act as contact (gustatory) receptors (Stephenson, 1979; Cook, 1985; Garraway, 1992; Dodds, 1997). When mollusks avoid certain plants, the semiochemicals act as toxins, deterrents, or antifeedants. For example, Airey et al. (1989) found that when the bicyclic monoterpenoid ketone (+)-fenchone, present in fennel, *Feoniculum vulgare* (Apiaceae), was applied to wheat seeds, it elicited a strong antifeedant response from the field slug *Deroceras reticulatum.* Subsequent work confirmed the neurophysiological activity of this compound, which induced intense electrical activity in the slug main olfactory nerve when applied to the appropriate chemoreceptor in the posterior tentacle (Garraway, 1992).

The Apiaceae (carrot family) comprising some 42 genera (Tutin, 1980) consists of annual or perennial herbs, rarely shrubs, many of which are strong smelling. In the work described, extracts of 33 species of Apiaceae were collected with a view to identifying the extract that elicited the greatest level of antifeedant activity against *D. reticulatum.* Individual extracts were applied initially to the slug olfactory chemoreceptor by using neurophysiological recording techniques, before being compared in complementary feeding bioassays.

METHODS AND MATERIALS

Test Animals. Adult *Deroceras reticulatum* (Müller) were collected from beneath refuge traps (cork/polystyrene roof insulation boards approximately 1 m^2) on an irrigated plot of mixed herbage. Slugs of known weight range (600–800 mg) were maintained in plastic bowls lined with moist cotton wool covered with absorbent, unbleached paper in a controlled environment room (12 hr light, 15°C; 12 hr dark, 5°C). The slugs were fed on Chinese cabbage (*Brassica chinensis*) grown from feed.

Test Materials. Foliage from 33 species of Apiaceae was tested (Table 1). Most species were grown from seed and maintained in glasshouses. The more common species, including *Anthriscus sylvestris, Heracleum sphondylium, Pimpinella major,* and *Chaerophyllum temulentum,* were collected on Rothamsted Farm. Samples of individual species were collected just prior to flowering and extracted on the same day. Extracts were stored in sealed glass vials (−20°C) until tested.

Extraction. Plant chemicals were extracted by using microwave-assisted dis-

TABLE 1. LIST OF SPECIES EXTRACTED FOR TESTING

No.	Species name
1.	*Petroselinum crispum* (parsley)
2.	*Pastinaca sativa* (wild parsnip)
3.	*Coriandrum sativum* (coriander)
4.	*Chaerophyllum temulentum* (rough chervil)
5.	*Anthriscus cerefolium* (curled chervil)
6.	*Conium maculatum* (hemlock)
7.	*Sium latifolium* (water parsnip)
8.	*Smyrnium olusatrum* (alexanders)
9.	*Crithmum maritimum* (rock samphire)
10.	*Myrrhis odorata* (sweet cicely)
11.	*Sison amomum* (stone parsely)
12.	*Bupleurum fruticosum* (hare's ear)
13.	*Angelica sylvestris* (wild angelica)
14.	*Berula erecta* (lesser water-parsnip)
15.	*Apium graveolens* (wild celery)
16.	*Falcaria vulgaris* (longleaf)
17.	*Aegopodium podagraria* (ground elder)
18.	*Seseli libanotis* (moon carrot)
19.	*Peucedanum officinale* (hog's fennel)
20.	*Anthriscus sylvestris* (cow parsley)
21.	*Oenanthe lachenalii* (parsley water-dropwort)
22.	*Heracleum sphondylium* (hogweed)
23.	*Daucus carota* (wild carrot)
24.	*Pimpinella major* (greater burnet saxifrage)
25.	*Hydrocotyle vulgaris* (marsh pennywort)
26.	*Scandix pecten-veneris* (shepherd's needle)
27.	*Ligusticum scoticum* (loveage)
28.	*Carum carvi* (carraway)
29.	*Physospermum cornubiense* (bladderseed)
30.	*Torilis japonica* (upright hedge parsley)
31.	*Atheusa cynapium* (fool's parsley)
32.	*Silaum Silaus* (pepper saxifrage)
33.	*Selinum carvifolia* (cambridge milk parsley)

tillation, based on the method of Craveiro et al. (1989). Approximately 30 g fresh weight of plant material was heated in a 500-ml florentine flask for approximately 1 min in an 800-W microwave oven until the plant cells ruptured. The volatile materials were picked up in a stream of nitrogen at a flow rate of 60 ml/min, carried through PTFE tubing (3 mm internal diameter), and trapped in a flask containing cooled hexane solvent. Connections between tubing and flasks were sealed with PTFE tape. Magnesium sulfate was added to remove water, then the extract was filtered. Extraction was confined to the leaves, as stems tended to burn, producing additional compounds not originally present in the plant.

Electrophysiology. The main olfactory organ of *D. reticulatum*, the posterior tentacle, contains two major nerves, the optic and the olfactory, both of which originate in the metacerebrum in the cerebral ganglion, with the olfactory nerve terminating in the digitate ganglion. The digitate ganglion divides into many smaller units until they become individual axons that terminate in sensory endings. These endings are interspersed between epidermal cells, giving the appearance of an epithelial pad, located immediately below the eye. A posterior tentacle was dissected out of an individual slug previously anesthetized by using CO_2 gas (Dodds, 1997). The dissected tentacle was placed in a Sylgard dish containing a specially formulated Ringer solution (NaCl 70 mM; KCl 2.5 mM; $CaCl_2 \cdot 6H_2O$ 3.5 mM; $MgCl_2$ 1.5 mM; $NaHCO_3$ 10 mM; KH_2PO_4 0.8 mM; $C_6H_{12}O_6$ 50 mM; pH 7.53) (modified from Garraway, 1992). Substitution of anhydrous $CaCl_2$ by $CaCl_2$ hexahydrate was found to increase the signal-to-noise ratio. The tentacle was dissected to further expose both the main olfactory nerve and the sensory pad by cutting away the retractor muscle and sheath. The preparation was positioned so that the nerve was immersed in the Ringer solution, while the sensory pad remained exposed to air so that airborne volatile stimuli could be applied.

Two suction electrodes containing Ag/AgCl wire were made (Garraway, 1992; Dodds, 1997). Periodically rechloriding the silver wire with a 1 M HCl solution increased the signal-to-noise ratio. The cut end of the nerve was drawn into the recording electrode (which had been trimmed to be a tight fit over the nerve) by suction generated by a 10-ml glass syringe, while the reference electrode was placed in the surrounding Ringer solution.

An airstream was purified by passage through activated charcoal and molecular sieves (0.5 μm) and humidified by passing through a gas jar containing water to prevent the preparation from drying out. A flowmeter was used to restrict the airflow to 60 ml/min, the maximum usable rate that did not stimulate firing in the olfactory nerve preparation (Garraway, 1992). The airflow passed continuously through a metal tube to an ejection port (1 mm diam.): 2-μl aliquots of test extracts were introduced into this airstream via an injection port and passed over the sensory pad. Extracts from all 33 species were screened on the same nerve preparation, with a 10-min resting period between dosing. Application of aliquots of (+)-fenchone (10 μg/ml) standard was implemented at random intervals to detect any desensitization of the preparation with time. During the course of the study, test chemicals were repeated on many different nerve preparations. Analysis was confined to the data obtained from the preparation that had been exposed to all 33 test extracts. Any electrical activity elicited in the tentacle preparation by the test stimulus was relayed through the electrode before being amplified (×1000) by a DAM50 differential amplifier (World Precision Instruments). Amplified electrical responses were simultaneously displayed onto a digital storage oscilloscope (Wavetek, 20 MHz) and recorded on magnetic tape for

subsequent computer analysis with appropriate software (Spike 2, Cambridge Electronic Design).

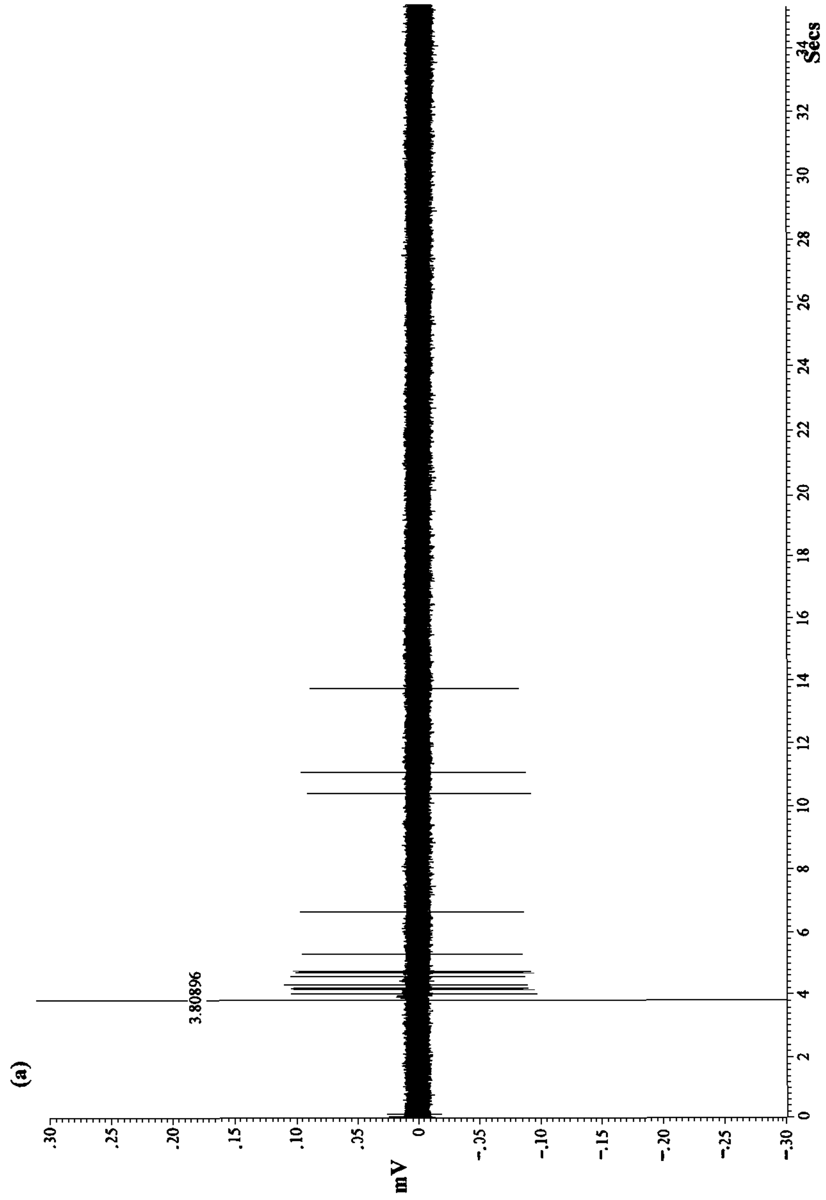
Wheat Flour Pellet Feeding Bioassay. This method is described in full in Clark et al. (1997); 30 μl of test extract was dosed onto 15 wheat flour pellets, and each pellet was exposed to a single *D. reticulatum.* The dry weights of individual pellets were measured before and after exposure to starved slugs for 24 hr. The weights consumed were analyzed by ANOVA, and the change in feeding (C), i.e., the difference in mean weights consumed by the test ($\overline{T}$) and control ($\overline{U}$) slugs, was expressed as a percentage with a standard error. As C is the ratio of two measured variables ($C = 100\ (\overline{T} - \overline{U})/\overline{U}$), and $\overline{T}$ and $\overline{U}$ are independent means, its standard error (SE) was calculated as SE $(C) = {}^{100}\surd[\{(\mathrm{Var}(\overline{T} + \mathrm{Var}(\overline{U})\}/\overline{U}^2 + (\overline{T} - \overline{U})^2\ \mathrm{Var}(\overline{U})/\overline{U}^4 + 2(\overline{T} - \overline{U})\ \mathrm{Var}(\overline{U})/\overline{U}^3]$ where $\mathrm{Var}(\overline{T}) = \mathrm{Var}(\overline{U}) = \surd(s^2/N)$, s^2 is the residual mean square from the analysis of variance, and N is the replication (here $N = 30$) (Stuart and Ord, 1987).

RESULTS

Electrophysiological Responses to Volatile Extracts. Of the 33 extracts from different Apiaceae species screened for activity, 22 triggered nervous activity in the slug olfactory nerve preparation. Intensity of response differed with plant species. Extracts such as *Anthriscus sylvestris* briefly induced a few APs, while those of species such as *C. sativum* and *C. maculatum* triggered an intense response lasting for over 60 sec (Figure 1). Several parameters were taken into consideration when comparing individual traces:

Duration of Response. Duration of the response was defined as the time in seconds during which activity remained above the spontaneous activity level. This was achieved by plotting a frequency histogram above each trace to identify exactly where the response started and finished. Frequency histograms were calculated for all 33 species. There were large differences in the duration of response, with *P. sativa* and *P. crispum* inducing large responses that lasted for well over 2 min, while the responses of *H. sphondylium* lasted only for 2 sec (Figure 2).

Total Number of Action Potentials (APs). In an intense response, e.g., when the extract of *C. maculatum* was applied to the olfactory nerve preparation, initial firing was rapid, producing up to ten spikes per second, and so computer software (Spike 2, CED) was used to calculate the exact number of APs produced during the response. There was a strong correlation between the duration of the response and the number of APs, with *P. crispum* extract producing the longest response with the greatest number of APs, and those of *H. sphondylium* producing the shortest response, with only three APs (Figure 3). However, the correlation was not complete; *C. sativum* extract, for instance, induced a relatively short response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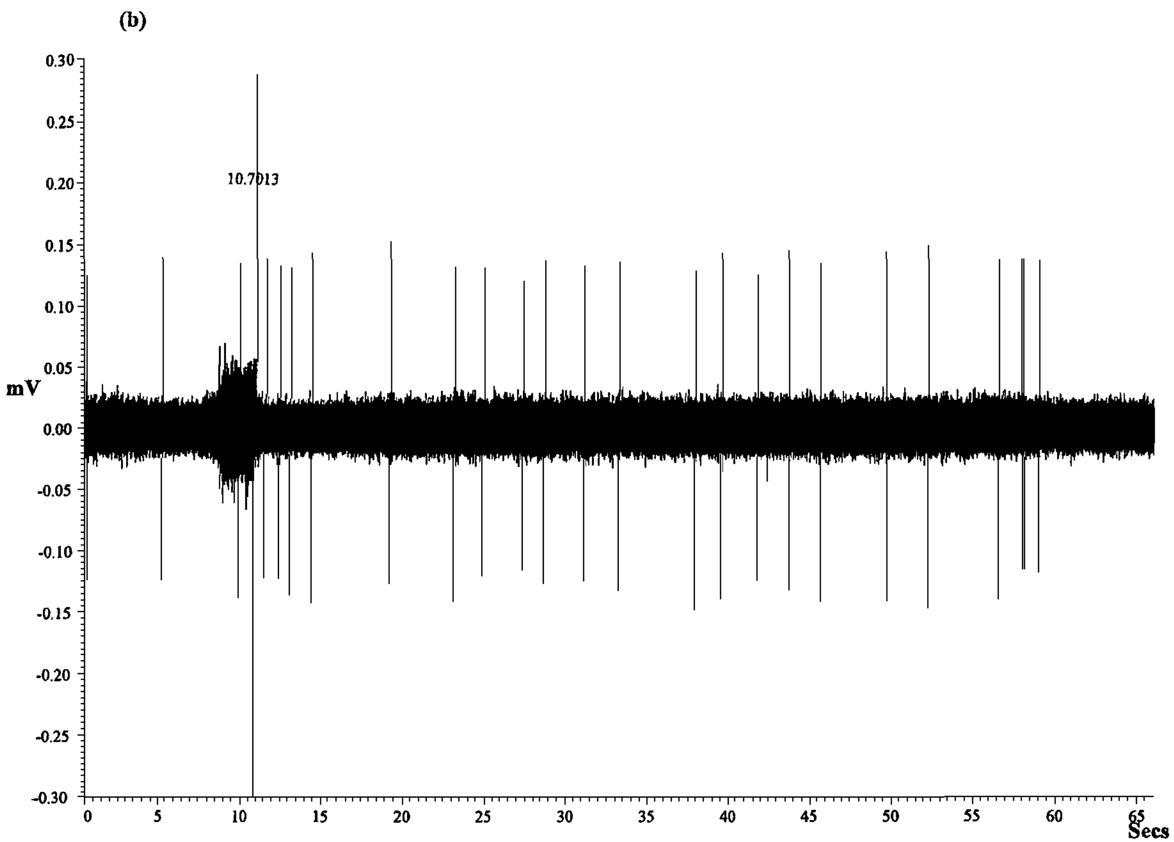
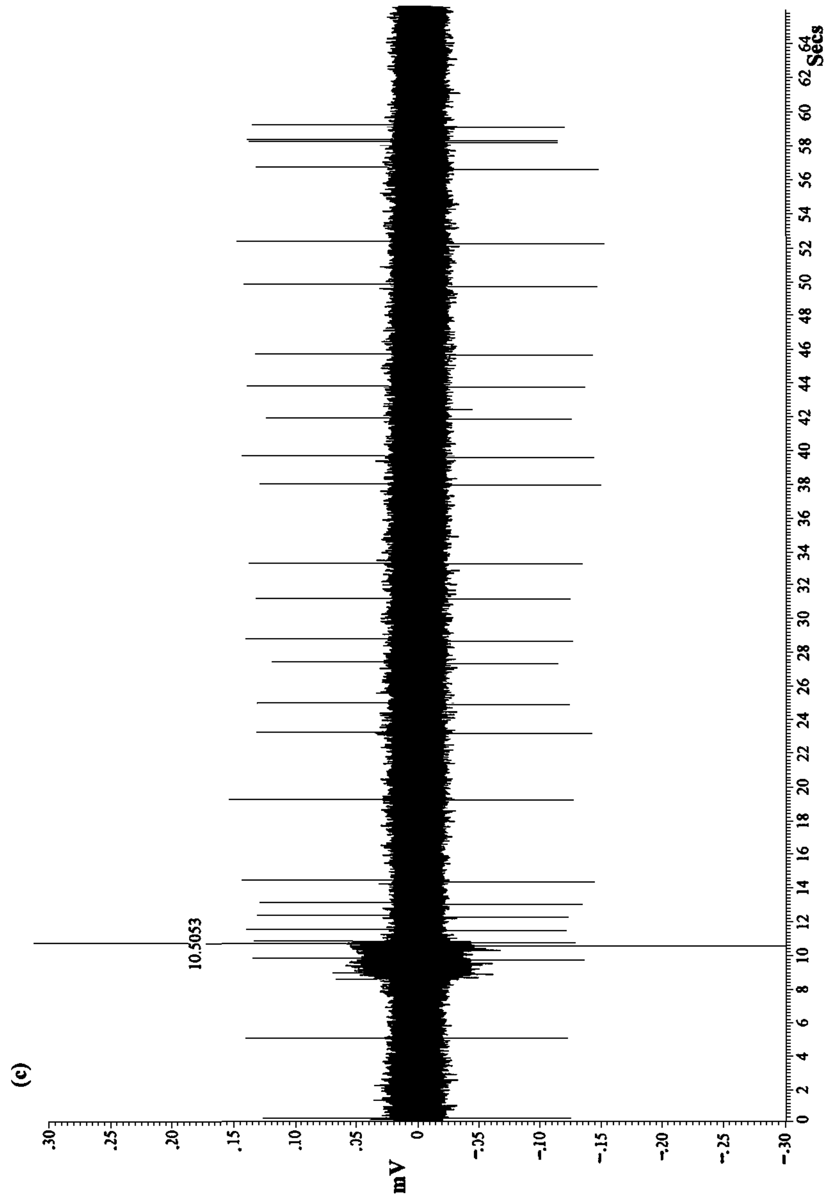


FIG. 1. Electrophysiological traces from the olfactory nerve preparation of *D. reticulatum*, in response to various extracts of Apiaceae. Cursors indicate where extracts were applied to tentacle nerve preparation. Extracts tested were: (a) *Anthriscus sylvestris*, (b) *Sison ammomum*, (c) *Myrrhis odorata*, (d) *Ageopodium podagraria*, (e) *Coriandrum sativum*, (*f*) *Anthriscus cerefolium*, and (g) *Conium maculatum*.


(c)
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mV
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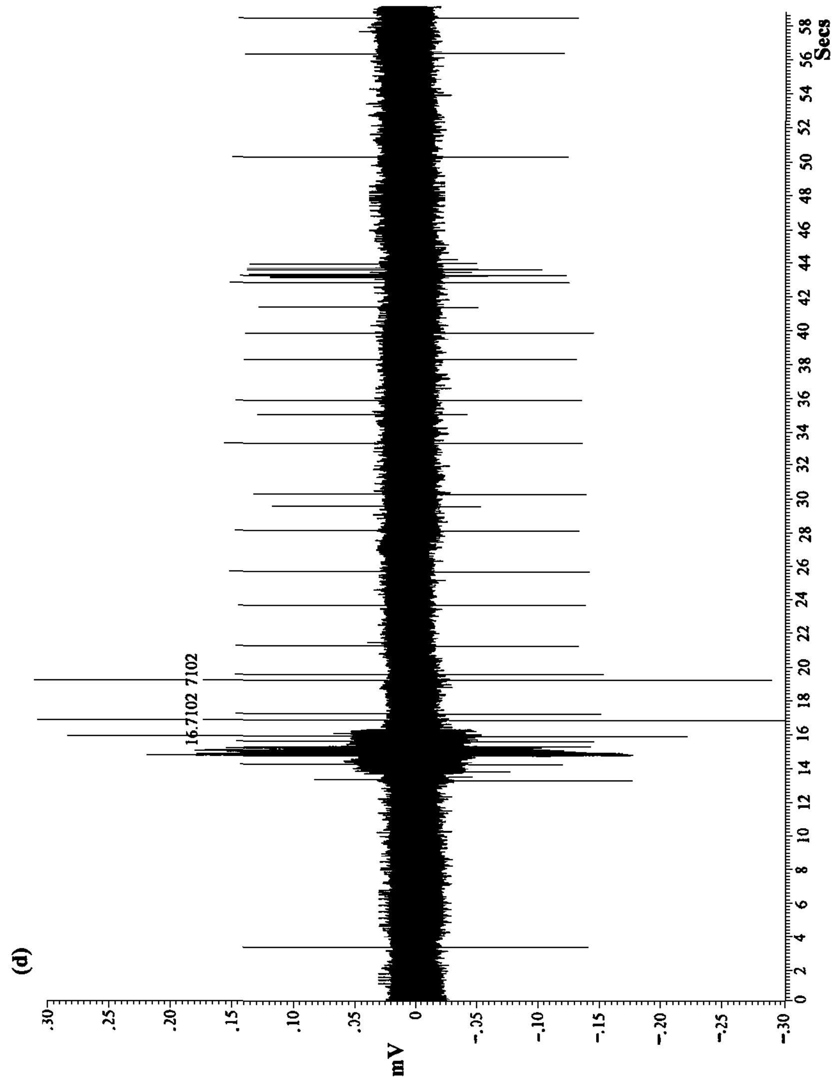
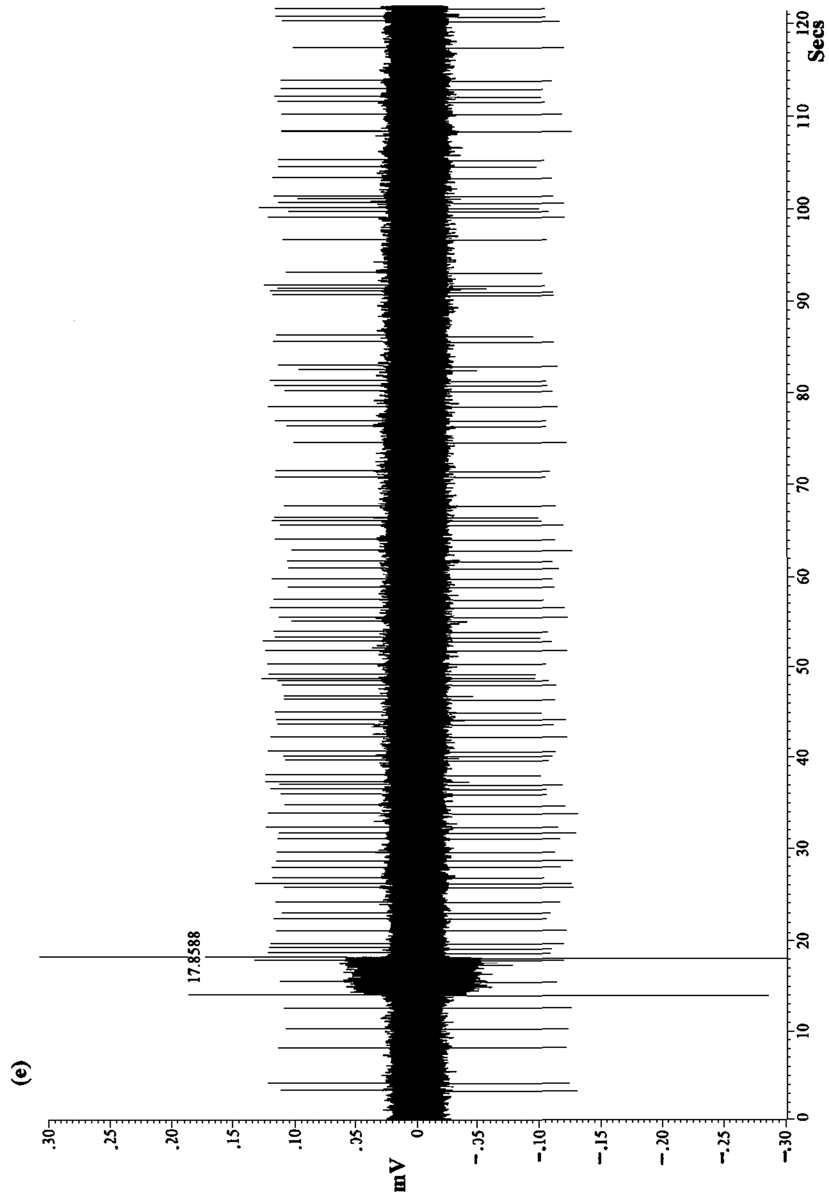


FIG 1. Continued


(e)
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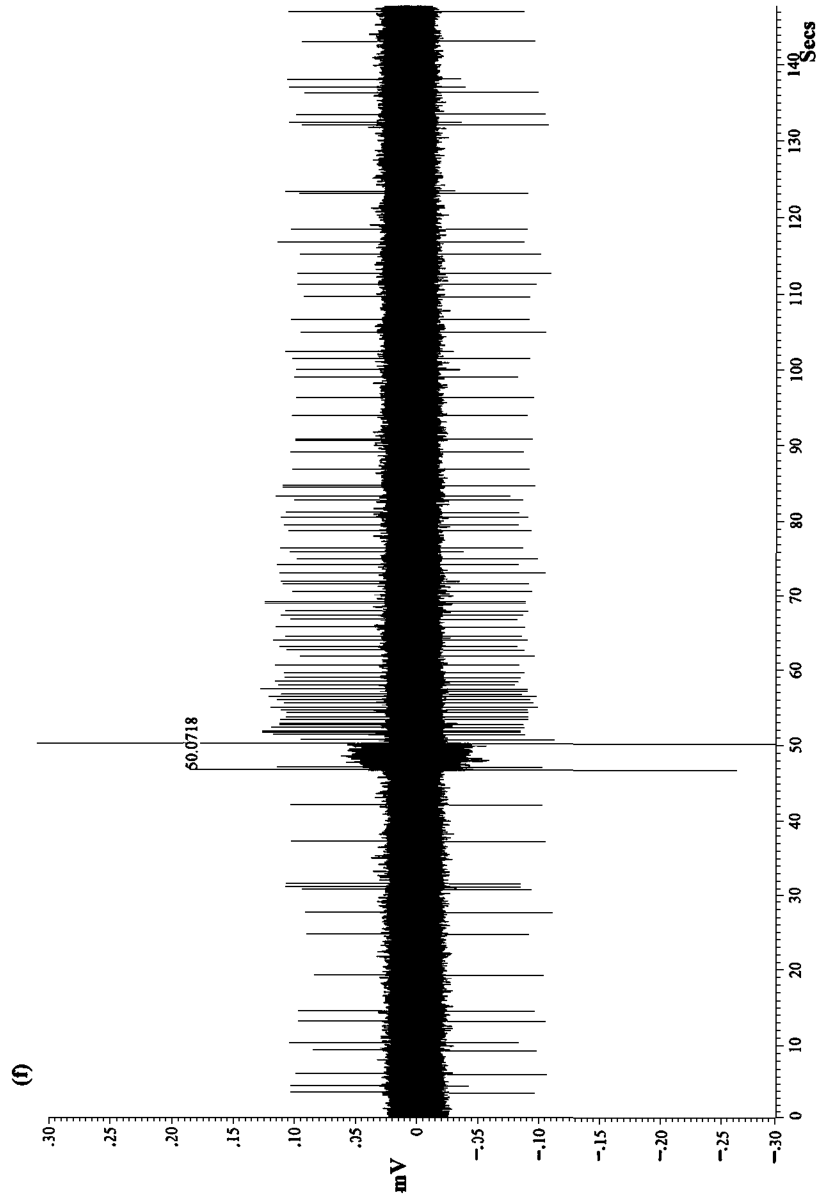

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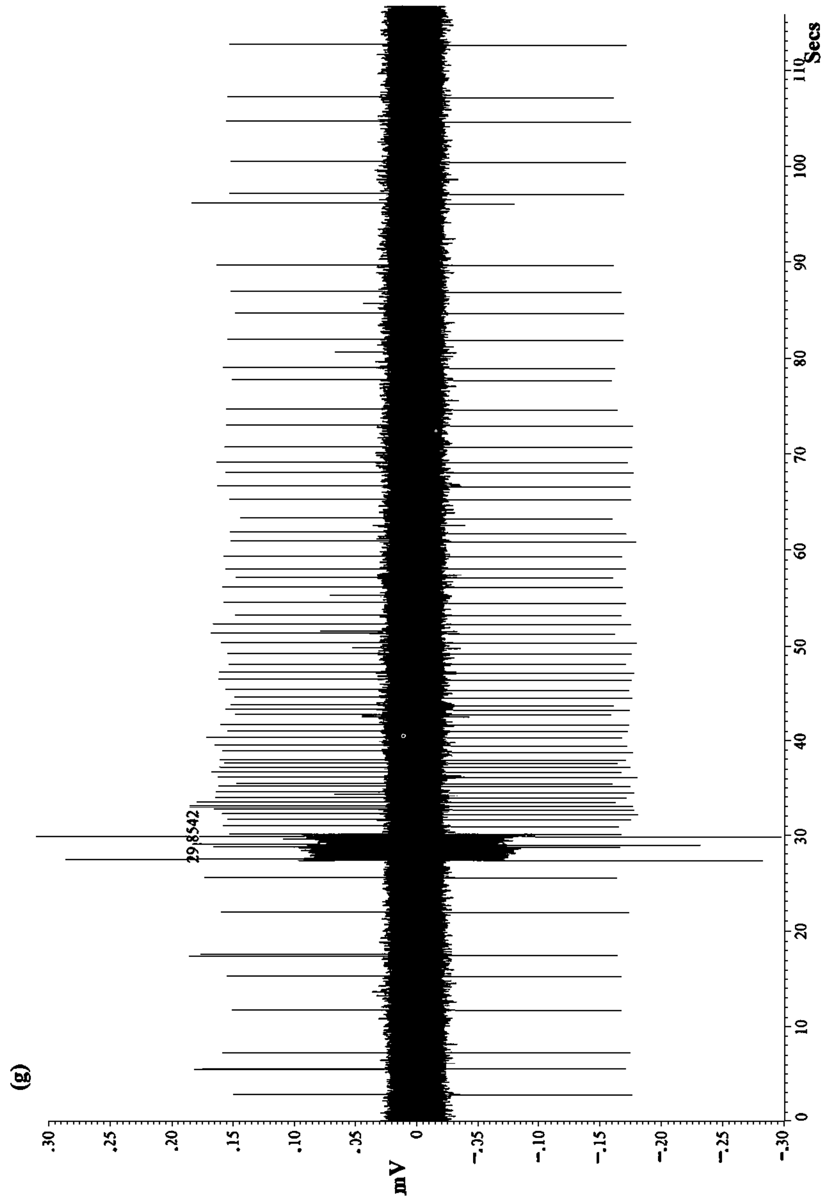

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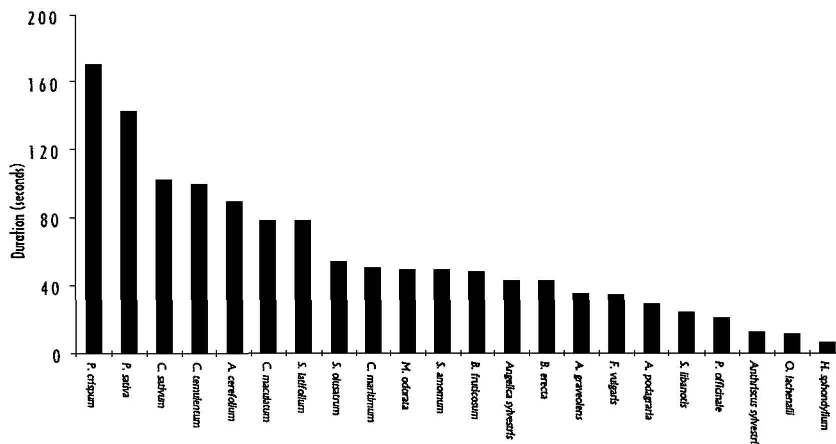

FIG. 2. Effect of volatile components from extracts of Apiaceae species on the electrophysiological activity of the olfactory nerve of *D. reticulatum:* Duration of response.

containing a large number of APs, whereas extract of *P. sativa* induced a longer response that actually contained only half the number of APs.

Increase in Frequency for the First Ten Seconds After Stimulation. When spontaneous activity levels were high before the test extract was applied, it was

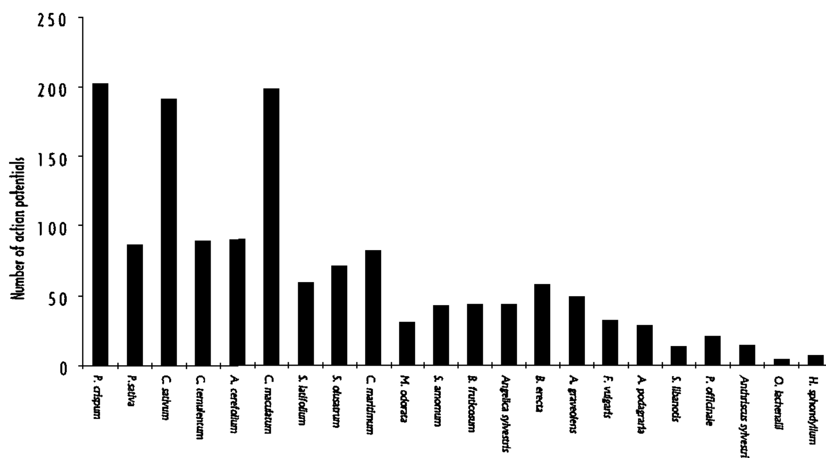

FIG. 3. Effect of volatile components from extracts of Apiaceae species on the electrophysiological activity of the olfactory nerve of *D. reticulatum:* total number of action potentials recorded following exposure.

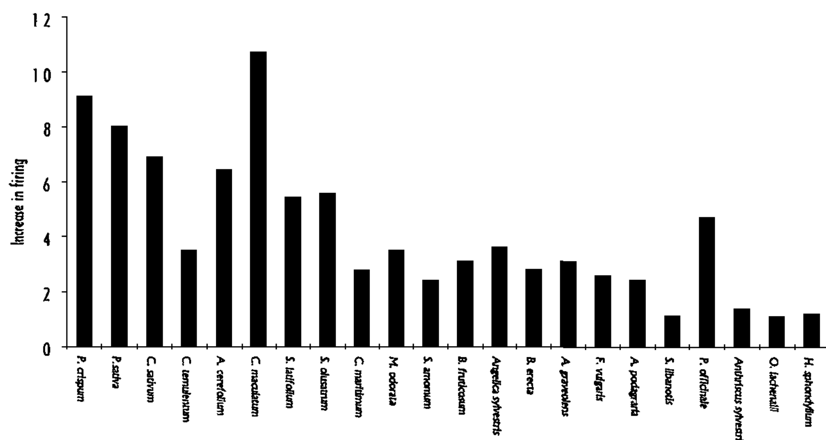

FIG. 4. Effect of volatile components from extracts of Apiaceae species on the electrophysiological activity of the olfactory nerve of *D. reticulatum:* increase in rate of firing during 10-sec period following exposure.

difficult to measure the changes in the frequency of the spikes produced in response to the extract. To overcome this, spike frequency was measured for approximately 50 sec prior to stimulation and then over a fixed period of 10 sec after the extract was applied with Spike 2 (CED). Any change in frequency of nerve firing during the 10-sec period was calculated for all active extracts (Figure 4).

Effects of Apiaceae Extracts on Feeding on Wheat Flour Pellets

The effect of extracts from different species of Apiaceae on feeding behavior in *D. reticulatum* is shown in Figure 5. Dotted lines on this figure separate species whose extracts are arbitrarily divided into five different categories of activity. Extracts from *C. maculatum* and *C. maritimum* reduced feeding by the largest amount and were located in the second category (80–60%). Extracts from the next set of species, including *A. cerefolium*, *P. crispum*, and *C. sativum*, all reduced feeding by a large amount (over 40%), while species such as *M. odorata*, *H. sphondylium*, and *P. cornubiense* reduced feeding to a lesser extent. The fourth group included species such as *S. silaus* and *A. cynapium*, which had little antifeedant activity, and *C. temulentum*, *P. major*, and *A. podagraria*, which had none.

Statistical Analysis

A principal components analysis (PCA) was performed to examine the variability between extracts. A data matrix was constructed for the 22 active extracts

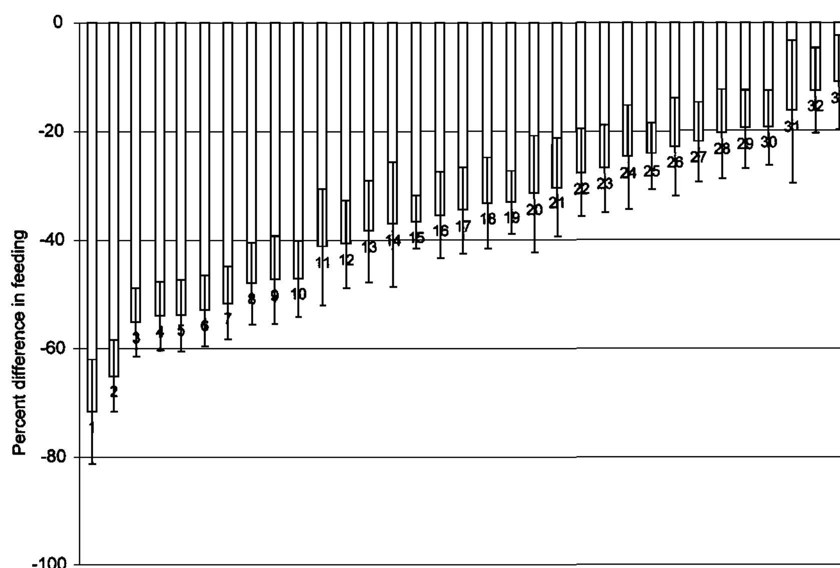

FIG. 5. Effect of adding extracts of Apiaceae species on weight of food eaten by *D. reticulatum* under standard conditions: 1, *Conium maculatum*; 2, *Crithmum maritimum*; 3, *Anthriscus cerefolium*; 4, *Pastinaca sativa*; 5, *Petroselinum crispum*; 6, *Smyrnium olusatrum*, 7, *Coriandrum sativum*; 8, *Bupleurum fruticosum*; 9, *Seseli libanotis*; 10, *Sium latifolium*; 11, *Oenanthe lachenalii*; 12, *Selinium carvifolia*; 13, *Myhrris odorata*; 14, *Heracleum sphondylium*; 15, *Physospermum cornubiense*; 16, *Angelica sylvestris*; 17, *Sison amomum*; 18, *Anthriscus sylvestris*; 19, *Peucedanum officinale*; 20, *Ligusticum scoticum*; 21, *Hydrocotyle vulgaris*; 22, *Apium graveolens*; 23, *Carum carvi*; 24, *Dacus carota*; 25, *Torilis japonica*; 26, *Falcaria vulagaris*; 27, *Berula erecta*; 28, *Scandix pecten-veneris*; 29, *Silaum silaus*; 30, *Aethusa cynapium*; 31, *Chaerophyllum temulentum*; 32, *Pimpinella major*; 33, *Aegopodium podagraria*.

for the four variables: (1) reduction in feeding due to extract (RF); (2) duration of nerve response induced in response to extract (Dur); (3) total number of APs produced in response to extract (NOE); and (4) increase in frequency of firing during first 10 sec after stimulation by extract (Freq).

The original frequency values were multiplied by a factor of 10 in order to increase their magnitude and range compared to that of the other three variables. If left unchanged, small differences in frequency values might have been concealed by differences in the other variables.

The latent vectors calculated indicated by v[2], v[3], and v[4] had coefficients in the first PCA dimension that were opposite in sign to that of v[1] and accounted for 87.4% of the variation. The PCA plot (Figure 6) illustrates that dimension I largely accounts for the separation of extracts 21,4, and 9 (*C.*

maculatum, *C. sativum*, and *P. crispum*, respectively) from the rest, indicating that they are dissimilar from each other and from the remaining species. These three species have high values for all four variables. There is also a clustering among extracts 1, 11, 8, 15, 19, 6, 16, 7, 2, 10, 12, 20, and 18. These numbers refer to the species *B. erecta*, *B. fruticosum*, *S. amomum*, *Angelica sylvestris*, *A. graveolens*, *M. odorata*, *F. vulgaris*, *A. podagraria*, *P. officinale*, *S. libanotis*, *Anthriscus sylvestris*, *O. lachenalii*, and *H. sphondylium*, respectively. These species had low values for each criterion. Dimension II, which is largely contributed by v[2] and v[3], contributes only 7.66% of the variation and accounts for most of the difference between *C. maculatum*, *C. sativum*, *P. crispum*, and *P. sativa*; the latter two both have higher v[2] values, and *P. sativa* had the lowest v[3] value of all four extracts.

DISCUSSION

Recordings of nerve responses to plant extracts were first recorded by Egan and Gelperin (1981), with extracellular recording from the tentacular nerve of the slug *Limax maximus*, when the posterior tentacle was exposed to extracts of carrot and mushroom. Garraway (1992) demonstrated electrophysiological activity in the tentacular nerve of *D. reticulatum* with a modification of this technique by cutting the nerve and sucking the entire length into the recording electrode; this increased the amplitude of the electrical signals recorded.

In the current study, when extracts of 33 species of Apiaceae were tested by using this method, 22 triggered nervous activity, inducing bursts of compound APs varying in duration and frequency. Extracts of species such as *C. maculatum*, *C. sativum*, and *P. crispum*, which induced intense electrical activity in the nerve preparation, also reduced feeding by up to 72% when incorporated in a standard food. The results demonstrate that the extracts contain compounds that are readily detected by the olfactory chemoreceptor in *D. reticulatum*, and compounds that are also strongly antifeedant, although they do not demonstrate whether both effects are caused by the same compound. In the group of plants that reduced feeding by at least 47%, the correlation between electrophysiological and antifeedant activity was noticeable, except in the cases of *C. maritimum*, *B. fruticosum*, and *S. libaonotis*. Extracts that reduced feeding by less than 25% were generally either inactive or produced only a low level of activity in the electrophysiological assay, while those with intermediate antifeedant activity showed no consistent pattern. Principal components analysis indicated that extracts of the three species, *C. maculatum*, *P. crispum*, and *C. sativum* differed from the other extracts, possibly implying a common active component.

Clapham et al. (1962) stated that there are some 43 genera of Apiaceae throughout Europe and that these can be grouped into various tribes or subtribes,

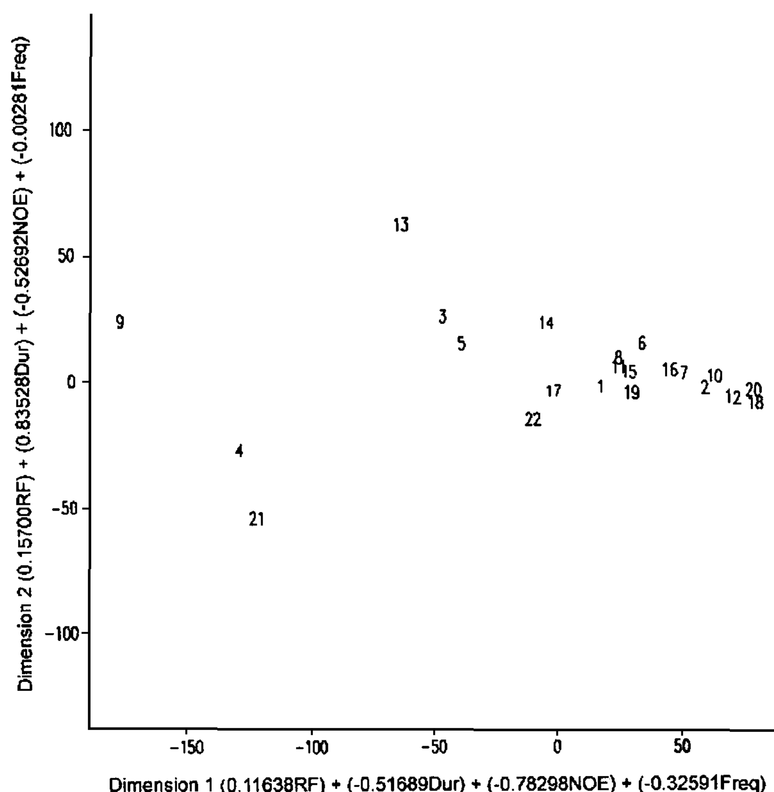

FIG. 6. Principal components plot illustrating differences in activity between 22 extracts of Apiaceae when neurophysiological data are combined with behavioural data. Clustering patterns illustrate clear changes in slug activity with different test extracts.

depending on their taxonomic similarities. No trends or similarities emerged when comparing the taxonomic groupings with the activity of the species in the electrophysiological and behavioral assays. For example, one particular tribe is said to contain three genera (*Conium*, *Smyrnium*, and *Physospermum*). *Conium* produced the most activity and, although *Smyrnium* did elicit a significant level of antifeedant activity, *Physospermum* was unable to trigger any activity in the electrophysiological preparation. A possible explanation for this difference in activity could be that although *Conuim* and *Physospermum* are similar morphologically, they are located in completely different habitats. *Conium* is found in damp areas such as open woods or river verges, whereas *Physospermum* can generally be found in grass verges (Clapham et al., 1962). As mollusks require damp conditions to prevent desiccation, it would seem that *Conium* may be sub-

ject to greater attack and, therefore, require some level of defense, in this case the presence of antifeedant chemicals released through the leaves. Clapham et al. (1962) also stated that the genera *Daucus* and *Seseli* are found in exposed areas with chalky soils. Due to this dry and exposed habitat, such an area would be unfavorable to shelless mollusks. Therefore, these two genera may be less likely to be attacked by slugs, which could explain the low activity found in the electrophysiological and behavioral tests.

Garraway (1992) concluded that compounds that elicited a repellent effect in behavioral tests were usually found in neurophysiological assays to produce spikes with a large average size, a large volume of response, and a high frequency of occurrence of spikes. The same relationship was found in this study where, in a range of species of Apiaceae, the most neuroactive extracts were also significantly antifeedant. Electrophysiological techniques may, therefore, give a useful indication of the antifeedant potential of naturally occurring compounds.

The neurophysiological screening assay combined with the complementary feeding bioassay is an efficient method for the rapid and extensive screening of plant extracts for potentially antifeedant activity. Active components within antifeedant extracts may be identified by using these same techniques coupled with GC-MS. This has been successfully achieved with several antifeedant extracts of Apiaceae and will be described elsewhere.

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DO PINE VOLES (*Microtus pinetorum*) USE NUMEROUS TYPES OF OLFACTORY CUES TO DISCRIMINATE GENDER?

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Abstract—Olfactory cues can contain information for discrimination of gender that can affect subsequent social interactions. Social rodents are hypothesized to use more olfactory cues than nonsocial rodents to distinguish males from females. The generality of this hypothesis was tested using the pine vole (*Microtus pinetorum*), a social vole. We examined nine possible sources of odors. A slide containing an odor from a male and a female was presented to each test subject for 3 min. We recorded the amount of time each test subject spent investigating each odor. Females spent significantly more time investigating male urine and male anogenital odors; however, they did not use any other odor sources to discriminate gender. In contrast, males did not use any odor sources to discriminate gender. Our results do not support the hypothesis that all social rodents use numerous odor sources to discriminate gender. Instead, our results are consistent with the alternative hypothesis that use of odor sources to convey information about gender may differ in rodents that live in different microhabitats.

Key Words—Vole, rodent, olfactory preferences, conspecific odors, sex differences, species differences, habitat differences.

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INTRODUCTION

In many species of animals, social behavior is influenced by olfactory signals. These olfactory cues may be involved in mate attraction, dominance interactions, use of space, or they may have physiological effects and elicit sexual or parental behavior (Brown and Macdonald, 1985; Halpin, 1986). Odor cues may provide information for recognition of species, gender, or individual identity, which can influence subsequent behavioral interactions.

Use of olfactory cues to discriminate gender has been shown in a variety of species including dwarf hamsters (*Phodopus sungorus campbelli*), in which males use scent markings to distinguish between males and females (Reasner and Johnston, 1987). In addition, secretions from sternal scent glands allow female Virginia opossums (*Didelphis virginana*) to recognize opposite-sex conspecifics (Holmes, 1992).

Wilson (1972) and Thiessen and Rice (1976) hypothesized that social species use a greater number of olfactory cues for discrimination of gender than do asocial species. There are few data available to test this hypothesis because, in the majority of studies, investigators have usually examined one or a few olfactory sources. An array of possible olfactory cues used in recognition has been examined in only a few species of mammals [golden hamster, *Mesocricetus auratus* (Johnston et al., 1993; Tang-Martinez et al., 1993); Djungarian hamster, *Phodopus campbelli* (Lai and Johnston, 1994; Lai et al., 1996); Norway rat, *Rattus norvegicus* (Natynczuk, 1990)] and a few species of voles (Ferkin et al., 1994; Ferkin and Johnston, 1995).

Ferkin et al. (1994) and Ferkin and Johnston (1995) tested 9–10 odor sources in two closely related species of arvicoline rodents, the meadow vole (*Microtus pennsylvanicus*) and the prairie vole (*M. ochrogaster*). Meadow voles are not social during the breeding season (Madison and McShea, 1987). Females have nonoverlapping home ranges (Madison, 1980) and exhibit aggressive behavior toward same-sex conspecifics. Home ranges of males overlap with each other as well as with the home ranges of multiple females (Madison, 1980). The mating system has been proposed to be promiscuity (Boonstra et al., 1993). Male meadow voles used urine, feces, and odors from the anogenital area, mouth, posterolateral region, and from nest material to discriminate gender (Ferkin and Johnston, 1995). Females showed a similar response, using odors from urine, feces, the anogenital area, the posterolateral area, and from nest material to distinguish between males and females.

In contrast, the closely related prairie vole is considered to be a social rodent (Getz et al., 1993). These animals live in male–female pairs or extended family groups. Males and females tolerate group members but are aggressive to other conspecifics (Getz et al., 1981). Male prairie voles used urine, feces, odors from

the anogenital area, the mouth, the posterolateral region, and the back to discriminate gender (Ferkin et al., 1994). Females used urine, feces, odors from the anogenital area, the mouth, the posterolateral area, the head-neck-ears area, and the chest to distinguish between males and females.

Comparison of the results obtained from these two species of arvicoline rodents led Ferkin et al. (1994) to speculate that social species, i.e., prairie voles, may use more sources of odor than less social species, i.e., meadow voles, to convey sexually distinct information to conspecifics. Therefore, the objective of our study was to identify the sources of sexually distinct odors in pine voles (*M. pinetorum*), a social species (FitzGerald and Madison, 1983; Dewsbury, 1990), and test the hypothesis of Ferkin and colleagues that, in arvicoline rodents, the number of gender-specific signals is greater in social than in asocial species.

Pine voles live in male-female pairs or in extended family groups of two to nine individuals (FitzGerald and Madison, 1983; Marfori et al., 1997; Solomon et al., 1998). DNA fingerprinting suggests that these animals are monogamous (Marfori et al., 1997). Pine voles are tolerant of group members but are aggressive toward unfamiliar conspecifics, particularly adults (Cranford and Derting, 1983). Males are significantly more aggressive toward adult males than toward adult females; females also are more aggressive toward same-sex conspecifics (Back, 1998). For these reasons, we expect that pine voles would show the ability to discriminate gender. Because pine voles are social, like prairie voles, we predicted that they use a similar number of olfactory cues to prairie voles and more odor sources than meadow voles for gender discrimination.

We also tested an alternative hypothesis, i.e., that species differences in the number of odors used to convey information about gender reflects differences in the habitat in which they live, the surfaces that they contact, and the substrates they encounter during routine activities (Alberts, 1992; Quay, 1968; Viitala and Hoffmeyer, 1985). Pine voles are semifossorial rodents found in a variety of habitats east of the Mississippi river (Smolen, 1981; Solomon and Vandenbergh, 1994) and are common pests in apple orchards where population densities may be higher than in natural habitats (Cornblower and Kirkland, 1983). In the latter, they construct extensive burrow systems under the driplines of trees (FitzGerald, 1984). If the use of gender-specific cues is related to microhabitat differences, then we would expect that pine voles would show a different pattern in use of olfactory cues than either meadow or prairie voles, both of which are terrestrial, although prairie voles also use underground nests (Wolff, 1985). A review of the sources of sensory communication in subterranean rodents suggests that olfaction may be less important than vibrational (vocal or seismic) communication (Francescoli, in press). If the semifossorial pine vole resembles subterranean rodents in the use of sensory modalities, then we predict that pine voles use fewer odor sources than prairie or meadow voles in gender discrimination.

METHODS AND MATERIALS

Animals. Voles used in this experiment were descendants of animals from the North Carolina State University colony and a small number of animals from an apple orchard in Henderson County, North Carolina. The colony was started in 1992 but, to prevent severe inbreeding, there were biannual additions of animals from the previously mentioned sources. Animals were maintained on a 14L : 10D schedule in a climate-controlled environment with a temperature of 25 ± 3°C. Lights came on at 06 : 00 hr EST.

The protocol used in this experiment was modified from that used by Ferkin et al. (1994) for prairie voles. Modifications from the previous study were made because pine voles mature more slowly than prairie voles. Test subjects were housed with both parents until 60 days of age in 36- × 30- × 18-cm plastic cages with paper bedding (Cell Sorb, A & W Products) and cotton (Nestlets, Ancare Corp.) for nesting material. Food (Purina Rodent Breeder chow) and water were provided ad libitum, and cages were cleaned every one to two weeks as needed. All pups born while test subjects were housed in the family cage were removed when they reached 24–28 days of age. When test subjects reached 60 days of age, they were housed individually in 24- × 18- × 14-cm cages, under the same conditions as were families. Test subjects were individually housed for three weeks to eliminate any effect of sibling presence until testing at 81 days of age, an age at which we could be sure that all animals were sexually mature. This age for testing of odor preferences was selected to correspond to similar studies of olfactory preferences of meadow and prairie voles (Ferkin et al., 1994; Ferkin and Johnston, 1995). Previous work has shown that sexual maturity of males and females is not delayed by housing with parents and younger siblings (Solomon, unpublished data).

Forty-four male and thirty-eight female pine voles were randomly chosen as test subjects. All test subjects were sexually inexperienced. Each vole was tested with as many as four different odor sources but all tests were at least five days apart as described by Ferkin et al. (1994) for prairie voles. Stimulus animals were sexually inexperienced adults that were weaned at 24 days of age and housed with littermates. An examination of ages from a subset of these animals showed that males ($N = 21$) were 84.7 ± 5.7 days of age and females ($N = 21$) were 85.6 ± 3.7 days of age when used to provide odor stimuli. One week before collection of the first odor cue, stimulus animals were housed in 18- × 29- × 12.5-cm cages with bedding and nest material. Food and water were provided ad libitum. Each stimulus animal was used for collection of up to five different types of odors. We expected that there would be natural variation in the production of odor cues and therefore used a unique combination of stimulus animals for each trial. Therefore, test subjects were never tested with the same stimulus animal more than once and care was taken to ensure that the stimulus

animals and the test subject were not related. Stimulus animals were randomly selected from the available pool of animals within these constraints. None of the females were in estrus.

Odor Testing Procedure. The odors presented to voles were fecal, urine, anogenital, head–neck–ears, back, chest, posterolateral, foot, and mouth odors. Odors were presented to test subjects, in random order, on clean glass slides (2.5 × 7.6 cm). We divided each slide into three equal sections, each 2.5 cm in length. One end of the slide contained an odor from a randomly chosen male or female stimulus vole (e.g., urine) and the opposite end contained the odor from the same source but from an opposite-sex conspecific. The middle portion of the slide contained no odor. We suspended the glass slide from a wire hook and clip attached to the middle section of the slide. The slide was suspended 2.5 cm above the bedding in the test subject's home cage, against the cage wall opposite the nest.

Testing began when the slide was placed into the test subject's cage. Only alert animals were tested. If an animal was sleeping in its nest, the test was postponed until a later time. Each trial lasted for 3 min from the time the slide was placed in the cage. For each trial, we recorded the time each test subject spent with the male and the female odor. We did not use the time the test subject spent investigating the middle section of the slide in data analysis. The criteria for investigation of an odor included the time the test subject spent licking or sniffing the stimulus odor and the time during which the test subject's nose was approximately 1 cm from the scented portion of the slide. Responsiveness to an odor was defined as investigation of at least one of the two odor cues.

Odor Collection. Unless otherwise noted, we conducted tests using fresh scents. Odors were collected from stimulus animals by rubbing the selected glandular area or body part for three to five seconds onto the appropriate side of the slide. The specific tissue sources of the odors from the anogenital area were not known and may have contained secretions from local sebaceous or apocrine glands, anal glands, and preputial glands; they also may have included contributions from urine, feces, and the penis or vagina. Fecal odor was collected by picking up a fresh bolus from the stimulus vole's cage with clean forceps and rubbing the bolus on the slide with a water-moistened cotton swab. Urine was collected by placing the stimulus animal on a screen in a small chamber for 1 hr, then freezing the urine at −4°C until needed. Thawed urine was rubbed onto the slide with a clean cotton swab. Mouth odor was collected by inserting a clean cotton swab, moistened with water, into the stimulus vole's mouth. The swab was then rubbed onto the randomly selected section of the slide. Slides were used only once and disposable gloves were worn while handling slides to prevent contamination with human odor.

Data Analysis. We used Wilcoxon matched-pairs signed-rank tests to compare time spent investigating male versus female odors. The possibility of posi-

tion effects, a preference for the left or right side of the slide irrespective of odor, was examined for each sex with binomial tests. Significant differences were accepted at $P < 0.05$.

RESULTS

Pine voles did not use many olfactory sources for gender discrimination. Females only used two odors for gender discrimination. They spent significantly more time sniffing male than female urine (Wilcoxon signed rank test: $z = 2.095$, $P = 0.036$) and male than female anogenital odors ($z = 2.194$, $P = 0.028$; Figure 1a). Females showed a tendency to prefer male to female chest odor ($z = 1.893$, $P = 0.058$) and female to male back odor ($z = 1.891$, $P = 0.059$; Figure 1b). Females did not show position effects (binomial test, two-tailed $P = 0.31$). The responsiveness of female pine voles ranged from 73% for fecal odors to 27% for foot odors (Table 1).

Males did not spend a significantly different amount of time sniffing any female or male odor (Figures 2a and b). Males also showed no position effects (binomial test, two-tailed $P = 0.22$). The responsiveness of males ranged from 71% for anogenital odors to 47% for foot odors (Table 1).

DISCUSSION

Although voles appear to use olfactory cues in numerous types of discriminations, the results of the current study show that female pine voles use only two olfactory cues, urine and anogenital odors, to distinguish gender. This is compared to female prairie voles and meadow voles, which used seven and four odor sources, respectively (Ferkin et al., 1994; Ferkin and Johnston, 1995). Our result argues against our first hypothesis, that the number of gender-specific odors used by social voles is greater than that used by asocial arvicoline rodents.

Female pine voles preferred male urine and male anogenital odors to odors from female conspecifics but did not use fecal odor to discriminate gender. These first two odor sources appear to be used in a number of species of voles [meadow voles, prairie voles, California voles (*M. californicus*), montane voles (*M. montanus*), and Townsend's voles (*M. townsendii*) (Quay, 1968; Jannett, 1981; Viitala and Hoffmeyer, 1985; Wolff, 1985; Ferkin et al., 1994; Ferkin and Johnston, 1995)]. When unfamiliar voles meet, they engage in mutual anogenital sniffing [e.g., prairie voles (Gavish et al., 1983)]. This behavior is seen in many species of voles and may be involved in recognition of species and gender as well as reproductive condition in the initial seconds of meeting. Furthermore, male olfactory cues in urine appear to be involved in reproductive activation of females in

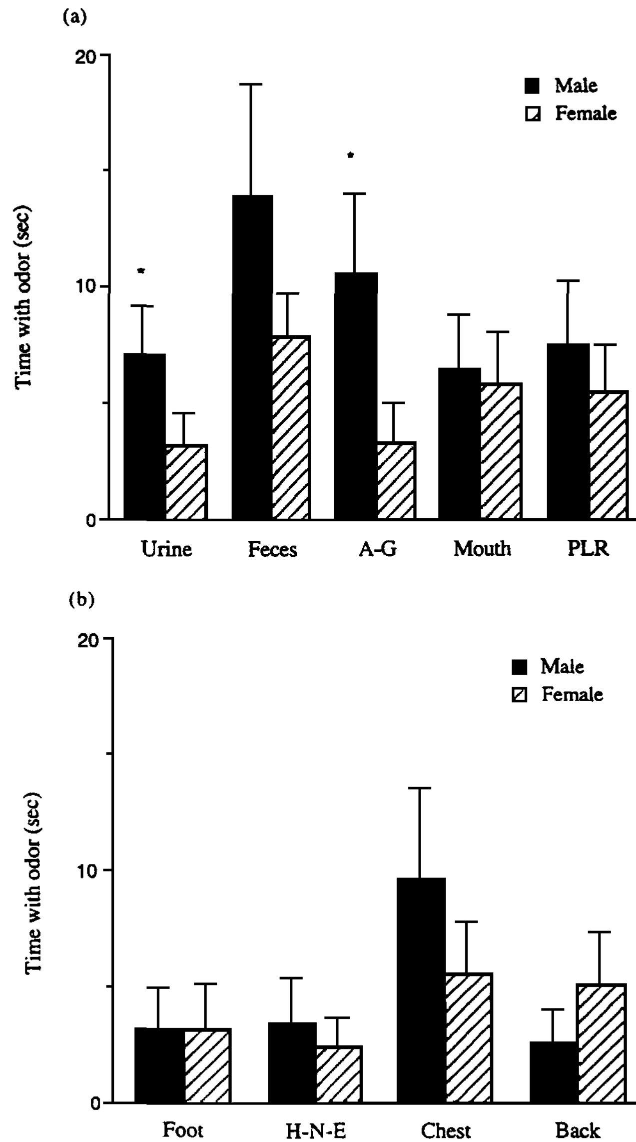

FIG. 1. (a) Length of time (mean ± SE) that female pine voles investigated male versus female odors from the following scent sources: urine, feces, anogenital region (A–G), mouth, and posterolateral (PLR); *significant difference between odor pairs. (b) Length of time (mean ± SE) that female pine voles investigated male versus female odors from the following scent sources: foot, head–neck–ears region (H-N-E), chest, and back.

TABLE 1. RESPONSIVENESS OF FEMALE AND MALE PINE VOLES TO ODOR PRESENTATION

	Number of Females responding		Number of Males responding	
Odor	*N*	%	*N*	%
Urine	8	53	11	64
Feces	11	73	10	67
Anogenital	9	60	12	71
Mouth	10	59	11	69
Posterolateral region	8	53	10	56
Head-neck-ears	5	33	11	65
Chest	7	44	9	56
Back	9	64	10	48
Foot	4	27	7	47

a number of species of arvicoline rodents (Richmond and Stehn, 1976; Sawrey and Dewsbury, 1985; Taylor et al., 1992).

Female pine voles showed a tendency to discriminate gender by using chest odor. This response is similar to that of female prairie voles (Ferkin et al., 1994) but contrasts with that of female meadow voles, which did not use chest odors to discern gender (Ferkin and Johnston, 1995). The lack of discrimination with respect to odors from the posterolateral area is not surprising because pine voles may have rudimentary posterolateral glands (Jannett, 1990). None of the females in these three species used foot odor to discriminate gender (Ferkin et al., 1994; Ferkin and Johnston, 1995; current study), although this odor might be used by bank voles, *Clethrionomys glareolus* (Griffiths and Kendall, 1980).

Although both females and males varied in their responsiveness to various odor cues, the variation was greater in females. For example, only 27% of females responded to foot odor as compared to 73% that responded to fecal odors. This variation suggests that some odors may be more salient to female pine voles than other odor cues.

Male pine voles did not use any of the nine olfactory cues to discriminate gender. This was not due to the lack of responsiveness to odors since, on average, 60% of male test subjects responded to each odor source. In contrast, male meadow and prairie voles used five and six olfactory cues to discern gender, respectively (Ferkin et al., 1994; Ferkin and Johnston, 1995). Clearly, males must distinguish between male and female conspecifics but may use olfactory cues in conjunction with those of another sensory modality or behavior in making that discrimination.

The results of the current experiment demonstrate that unlike another social species, the prairie vole, pine voles do not use more odor sources in gender discrimination than does one asocial species, the meadow vole. In a previous study,

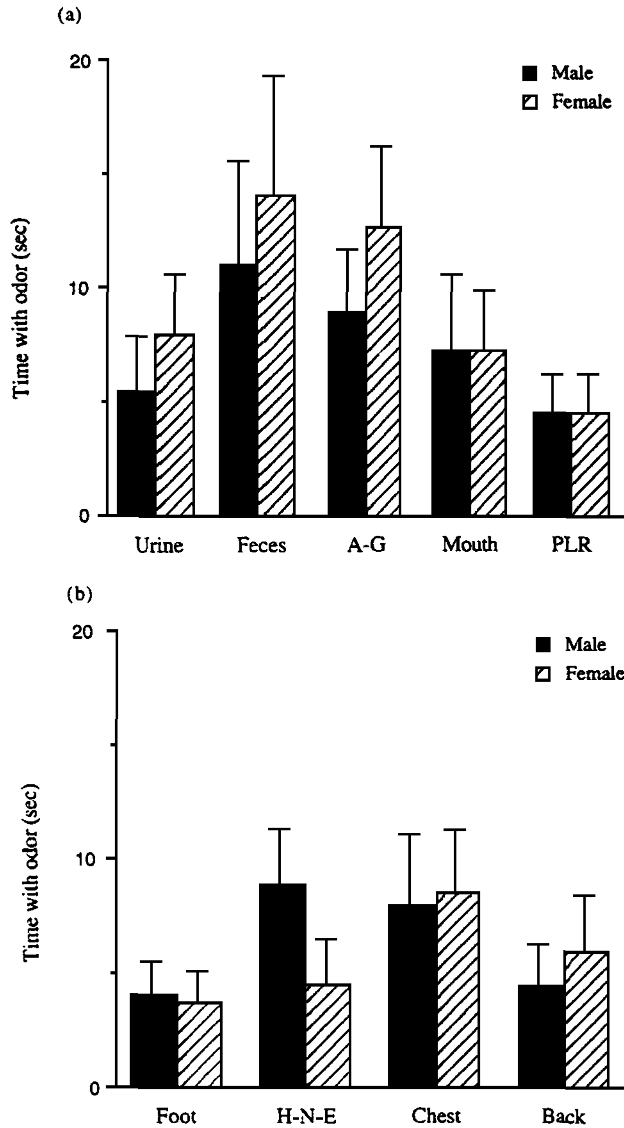

FIG. 2. Length of time (mean ± SE) that male pine voles investigated male versus female odors from the following scent sources: urine, feces, anogenital region (A-G), mouth, and posterolateral (PLR). (b) Length of time (mean ± SE) that male pine voles investigated male versus female odors from the following scent sources: foot, head–neck–ears region (H-N-E), chest, and back.

Solomon (1999) reported that adult female pine voles did not spend different amounts of time with unfamiliar male versus unfamiliar female bedding during a 24-hr test. This result is consistent with the finding in the current study that females do not use as many odors as previously examined arvicoline rodents to discriminate gender. One possible reason for the differences among the three species could be that the pine vole does not have as many glands as the other two species or that their glands are not as well developed. Jannett (1986) reports that vesicular and scent glands of prairie voles accounted for 18% of their body weight; in meadow voles these glands accounted for 15% of their body weight. In contrast, the glands in the pine voles accounted for only 12% of their total body weight and some glands, e.g., posterolateral glands, are rudimentary or absent (Jannett, 1990). Although these data are suggestive, it is not known if these percentages are significantly different among species. Even so, if production of odors is related to the size or number of glands, we expect that prairie vole odor production would be greater than that of meadow voles, which in turn would be greater than that of pine voles. If this is true, it may explain why pine voles use fewer sources to discriminate gender than the other two species.

We think that our results are best explained by our alternative hypothesis, which is that species differences may reflect adaptation to diverse environmental features in their habitats (Alberts, 1992; Quay, 1968; Viitala and Hoffmeyer, 1985). It is possible that pine voles do not need to use as many olfactory cues to distinguish between males and females because odors dissipate more slowly in burrows. The information available on sensory ecology of subterranean rodents supports the view that olfaction is not likely to be the primary means of communication in animals that spend the majority of their time underground. Olfaction is not used to as great an extent as are vocal or seismic signals in many subterranean species, and those olfactory cues that are used tend to be urinary odors (Heth et al., 1996; Francescoli, in press). Tactile cues appear to be important in taxa of social subterranean rodents (Francescoli, in press) and have been proposed to be necessary for some types of intraspecific communication in pine voles (e.g., a combination of chemical cues and contact with an unfamiliar male is necessary for reproductive activation of female pine voles) (Solomon et al., 1996). Pine voles also may rely on odors less than on physical interactions in gender discrimination.

In conclusion, it appears that some social rodents, such as pine voles, do not use numerous odor cues to distinguish gender. Our data support the hypothesis that the number of sources of gender-specific scents available to an animal reflects its microhabitat and whether it is terrestrial or fossorial. Examination of the use of olfactory cues in additional species of arvicoline rodents is necessary to verify these results.

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TOXICITY OF DANGEROUS PREY: VARIATION OF TETRODOTOXIN LEVELS WITHIN AND AMONG POPULATIONS OF THE NEWT *Taricha granulosa*

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Abstract—The ability to identify and accurately measure traits at the phenotypic interface of potential coevolutionary interactions is critical in documenting reciprocal evolutionary change between species. We quantify the defensive chemical trait of a prey species, the newt *Taricha granulosa*, thought to be part of a coevolutionary arms race. Variation in newt toxicity among populations results from variation in levels of the neurotoxin tetrodotoxin (TTX). Individual variation in TTX levels occurs within populations. Although TTX exists as a family of stereoisomers, only two of these (TTX and 6-epi-TTX) are likely to be sufficiently toxic and abundant to play a role in the defensive ecology of the newt.

Key Words—*Taricha granulosa*, Caudata, coevolution, tetrodotoxin, predator–prey, arms race.

INTRODUCTION

Reciprocal selection and evolutionary change between two or more taxa are fundamental to the concept of coevolution (Janzen, 1980; Futuyma and Slatkin,

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1983; Vermeij, 1994; Thompson, 1994). Demonstrating this selection requires evidence that sufficient heritable variation exists in the relevant traits for both species to respond to selection and that changes in the trait values of one species directly drive changes in trait values of the partner species (Berenbaum and Zangerl, 1992, 1998). The primary requisite for obtaining evidence of reciprocal evolution is the ability to identify and quantify the specific traits that mediate the interaction between species.

When the characters at the phenotypic interface of coevolution are morphological or behavioral, this task is fairly straightforward. When the interaction involves a defensive chemical and corresponding resistance, investigating the core traits of the relationship is more problematic. Chemically mediated coevolution is an ideal context in which to document reciprocal evolutionary change (Spencer, 1988), but practical problems associated with its study have confounded progress. These difficulties are exemplified in plant–herbivore systems, where identifying the most critical defensive trait or traits is obfuscated by the diversity of potential defensive compounds that can be found in a single plant (Berenbaum and Zangerl, 1992; Rosenthal and Berenbaum, 1991; Bernays and Chapman, 1994). Phytophagous insects probably respond to suites of chemicals rather than individual compounds, further complicating the task of determining which chemicals are relevant to a given interaction (Berenbaum and Zangerl, 1992). Similarly, many putative defensive compounds can affect a broad range of exploiting taxa (Berenbaum and Zangerl, 1992). Determining the species composition of a given coevolutionary interaction can be difficult when this range of effect is coupled with the varying degrees of specialization present in phytophagous insects (Berenbaum and Zangerl, 1992; Bernays and Chapman, 1994; Thompson, 1994). Similar problems exist in chemically mediated exploitive interactions between animals. Predator–prey interactions usually involve multiple participants and animals also can produce a myriad of potential defensive compounds (Daly et al., 1984, 1987).

Even when the relevant species and chemicals are identified, the logistics of quantitatively sampling target chemicals is troublesome. Behavioral mechanisms allow many insect herbivores to reduce their exposure to defensive compounds (reviewed in Bernays and Chapman, 1994). Similarly, many predators have developed patterns of behavior allowing them to avoid prey toxins [e.g., shrikes and grasshoppers (Yosef and Whitman, 1992), raccoons and toads (Groves, 1980)]. The results of these behaviors are that the estimates obtained by a scientist sampling whole leaves or skin sections in the laboratory can differ widely from the actual concentrations experienced by the organisms being studied. Finally, errors in estimates of chemical concentrations can arise because of variance in toxicity assays (Martin and Martin, 1982).

An example of a chemically mediated exploiter–victim system that is not hampered by the above complications is the interaction between the toxic newt

Taricha granulosa and its resistant predator, the garter snake *Thamnophis sirtalis* (Brodie and Brodie, 1990, 1991). Unlike most insect–plant systems, the taxa that comprise the interaction are few and well understood. Only one predator (*Thamnophis sirtalis*) and a single genus of prey (the salamandrid genus *Taricha*) are involved, and the traits at the phenotypic interface have been clearly identified (Brodie and Brodie, 1990, 1991). Newts of the genus *Taricha* possess the potent neurotoxin tetrodotoxin (TTX) (Mosher et al., 1964; Wakely et al., 1966; Brodie et al., 1974; Yotsu et al., 1990), and *Taricha granulosa* is the most toxic species of the genus (Brodie et al., 1974). Tetrodotoxin is a low-molecular-weight, nonprotein toxin found in a large number of disparate taxa. Its mode of action is well understood; TTX binds to and blocks Na^+ channels, thereby interfering with the propagation of nerve and muscle action potentials. This system is further simplified by the fact that TTX is unlikely to have synergistic interactions with its stereoisomers. Tetrodotoxin and tetrodotoxin analogs compete for the same binding site in Na^+ channels, and analogs with low toxicity relative to TTX appear to have lower binding affinity for the binding site in known systems. (Kao and Walker, 1982; Kao and Yasumoto, 1985; Yang et al., 1992).

Tetrodotoxin is known to be lethal to most potential predators (Brodie, 1968) and is an important antipredator mechanism for *Taricha granulosa* (Brodie, 1968; Brodie et al., 1974; Daly et al., 1987). *Thamnophis sirtalis* is the only known potential predator of *Taricha granulosa* that can survive ingestion of TTX (Brodie, 1968). This species has evolved resistance to TTX, allowing it to exploit newts in regions where the two are sympatric (Brodie and Brodie, 1990). Although *Taricha granulosa* also may posses an additional high-molecular-weight toxin (Brandon and Huheey, 1981, 1985), most of their toxicity likely results from TTX and its stereoisomers (Brodie et al., 1974). Additionally, because snake predators swallow their prey whole, they are unable to perform the sort of limited sampling of prey chemicals that complicates the relationship between the amounts of compounds found in prey and the amounts experienced by predators.

The limited number of defensive chemicals in this system makes the identification of the relevant defensive trait (TTX toxicity) straightforward, but quantifying variation in the trait has been more problematic. Previous studies have examined total toxicity of newts rather than TTX levels (Wakely et al., 1966; Brodie et al., 1974; Brodie and Brodie, 1991). This inability to quantify TTX itself has prevented investigation of the linkage between the defensive trait (TTX) in prey and the exploitative trait (TTX resistance) of predators.

In this study we used high-performance liquid chromatography (HPLC) techniques to quantify the TTX levels of *Taricha granulosa* from seven populations in the Pacific Northwest of North America. High-performance liquid chromatography provides an accurate and highly sensitive assay for TTX and TTX analogs (Yasumoto and Michishita, 1985; Yotsu et al., 1989; Wu et al., 1996). This technique has been used to study TTX in *Taricha granulosa* and

other salamandrids (Yotsu et al., 1990; Kotaki and Shimizu, 1993), as well as other Amphibia (Yotsu-Yamashita et al., 1992; Daly et al., 1994, 1997; Mebs et al., 1995). We tested the hypothesis that previously documented population variation in newt toxicity results from variation in TTX levels by sampling from populations where total toxicity had already been assayed (Brodie, 1968; Brodie et al., 1974). In addition, we explicitly tested the hypothesis that populations of *Taricha granulosa* from Vancouver Island, British Columbia, do not possess TTX (Brodie and Brodie, 1991) by sampling populations from this area. We also sampled three other populations from Oregon and Washington to investigate patterns of interpopulational variation in TTX levels of *Taricha granulosa*. Finally, because multiple analogs of TTX have been found in *Taricha granulosa* (Yotsu et al., 1990; Kotaki and Shimizu, 1993), we examined the stereoisomers of TTX to determine which analogs are both toxic and abundant enough to be biologically important in the interaction between *Taricha granulosa* and *Thamnophis sirtalis*.

METHODS AND MATERIALS

Samples. Seventeen adult *Taricha granulosa* were collected from six sites in Oregon, Washington, and the Vancouver Island region during 1996 and 1997 (Figure 1). Specimens from Oregon collected in 1996 or 1997 came from one of two populations: a coastal population in Lane County [Tenmile ($N = 5$)], and a Willamette Valley population [Benton County ($N = 1$)]. Specimens from Washington came from a coastal site [Grays Harbor ($N = 4$)] and the Olympic Peninsula [Beaver Lake ($N = 4$)]. Of the animals collected on or around Vancouver Island, one site is on Vancouver Island [Qualicum ($N = 1$)]; the other site is on an island adjacent to Vancouver Island [Texada Island ($N = 2$)]. These animals were either frozen immediately at −80°C or tissue was removed and frozen in liquid nitrogen. Preserved specimens from two sites [Reid Island, British Columbia ($N = 4$), and Benton County, Oregon ($N = 4$)] were also used. These preserved animals were collected in 1989, preserved in 95% ethanol and stored at 4°C. Preserved animals from Reid Island, the third site from the Vancouver Island region, came from an island near the southern end of Vancouver Island. Because preserved and wild-caught animals from Benton County were from physically proximate sites, samples from these two groups were pooled as "Benton County." Previous research on TTX (Brodie, 1968; Brodie et al., 1974) indicated that TTX toxicity was stable for at least one year when stored at low temperatures. Data collected for this manuscript indicated that samples from the same population (Benton County) did not significantly differ in TTX levels regardless of their method or length of storage (fresh frozen vs. storage in ethanol for eight years), indicating that neither the length nor method of tissue storage in this study altered

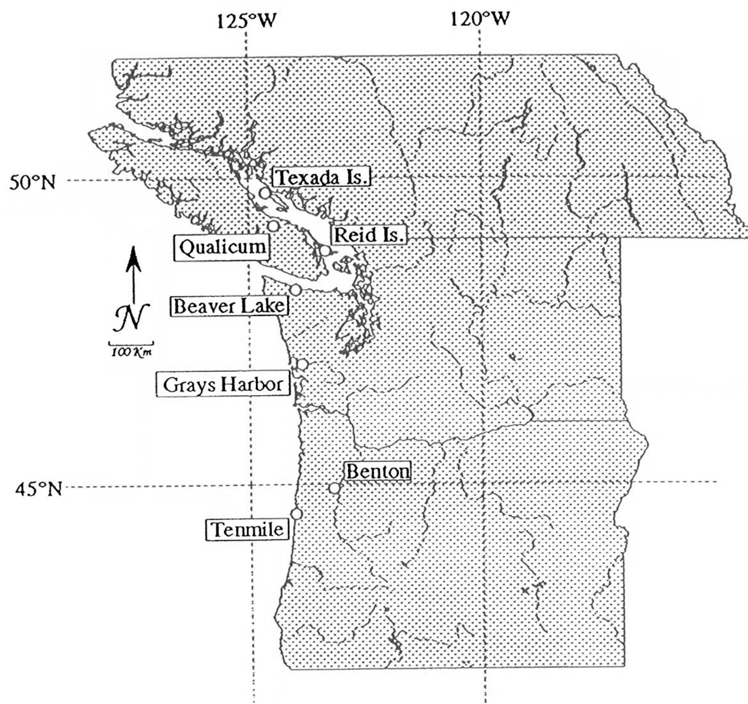

FIG. 1. Map of populations surveyed.

TTX levels. Therefore, frozen and preserved samples for any given population were pooled in this study.

Toxin Extraction. Extracts from each sample were prepared from dorsal skin that had either been removed from subjects and immediately frozen at −80°C (specimens from 1996 or 1997) or removed from subjects just prior to toxin extraction (preserved specimens from 1989). Each tissue sample was weighed and cut into small pieces, and enough extraction buffer (0.1 M aqueous acetic acid) was added to bring the final concentration of the sample to 0.05 g skin/ml of buffer. Samples were heated in a boiling water bath for 5 min and 1 ml of the resultant extract spun at 10,000 rpm for 10 min. Following this first spin, 0.5 ml of the supernatant was spun at 10,000 rpm for 10 min in 0.5-ml Millipore centrifuge filter tubes (Ultrafree-MC, 10,000 NMWL filter unit). Aliquots of 10 μl of this final filtrate were used for analysis. Repeatability of the extraction protocol was measured with two independent skin extractions on individuals from Tenmile ($N = 5$). Each of these extractions was analyzed as an independent sample, and the repeatability of the extraction was calculated using a one-way

ANOVA (Becker, 1992). The repeatability for this extraction protocol and toxin analysis was high ($r = 0.95$), indicating that the extraction protocol was not a significant source of variance.

TTX Assay. The levels of TTX and TTX analogs present in newts were quantified by fluorometric HPLC (Yasumoto and Michishita, 1985; Yotsu et al., 1989). Separation of analogs was performed on a Develosil ODS-5 column (0.46 × 25 cm, Nomura Chemical, Seto, Japan) with a 50 mM ammonium acetate and 30 mM ammonium heptafluorobutyrate buffer (pH 5.0) containing 3% acetonitrile run at a flow rate of 0.5 ml/min. The eluate was mixed with an aqueous 4 N NaOH solution from another pump (1.2 ml/min), and passed through a stainless steel tube (0.5 mm × 10 m) heated at 105°C to derive analogs to fluorophore. After cooling in a 30-cm water jacket, the fluorescent derivatives were detected by a Jasco FP110 fluoromonitor (Jasco Co. Ltd., Tokyo, Japan). The excitation wavelength of the detector was set at 365 nm and emission wavelength set at 510 nm. The peak areas were measured and integrated with a Hitachi Chromato-Integrator D-2500.

Standards and Analysis. Standard solutions of TTX, 4-epi-TTX, and 4,9-anhydro-TTX were isolated from pooled eggs of *Fugu poecilonotus* and *F. pardalis* (Nakamura and Yasumoto, 1985). The 6-epi-TTX standard was isolated from the newt *Cynops ensicauda* (Yasumoto et al., 1988). The purity of these compounds was checked by a combination of HPLC, thin-layer chromatography (TLC), ^{1}H NMR spectroscopy, and mass spectrometry (Yotsu et al., 1990). Tetrodotoxin was quantified by weighing. Because of the difficulty of weighing small amounts of hydroscopic TTX isomers, quantification was made by ^{1}H NMR spectroscopy with TTX as the standard (Yotsu-Yamashita, et al., unpublished data). Because the HPLC analyzer is 20 times more sensitive to 6-epi-TTX than to TTX (Figure 2) (Yasumoto and Michishita, 1985; Yotsu et al., 1989), a standard solution of 6-epi-TTX was prepared separately from the standard mixture of TTX, 4,9-anhydro-TTX, and 4-epi-TTX. This difference in sensitivity also means that direct comparison of peak area or height for different analogs (Figure 2) is not an accurate method of gauging the relative amounts of each analog. Therefore, the amount of each analog was calculated based on the standard curve for each analog. This relationship is linear (Yasumoto and Michishita, 1985; Yotsu et al., 1989) within the ranges of TTX and 6-epi-TTX of our samples and could be used to quantify the amount of TTX and 6-epi-TTX present in our samples. The lower limit of detection of this system is 4.5 μg of TTX per gram of skin and 0.23 μg of 6-epi-TTX per gram of skin. The analyzer was calibrated by injecting the TTX and 6-epi-TTX standards before and after assaying each sample. All samples were assayed twice and the mean of those measures used for analysis.

Statistical Analysis. Because our raw data were heteroscedastic and had variances proportional to group means, we used a square root transformation on

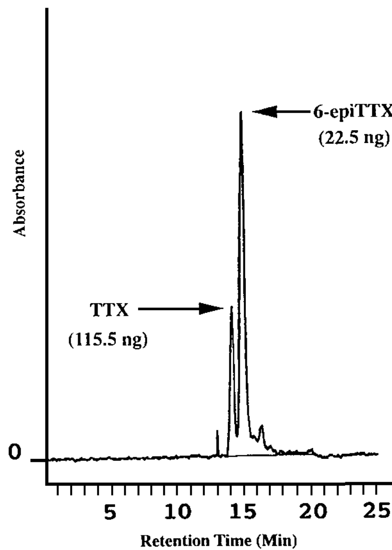

FIG. 2. HPLC chromatogram of TTX (115.5 ng) and 6-epi-TTX (22.5 ng) standards showing the differential sensitivity of the analyzer. The area under each peak is linearly related to the quantity of that compound. Note that the scale of this figure is different from that of other figures and cannot be used for direct comparison of TTX and TTX analog concentrations.

our data (Zar, 1984). These transformed data were used for all analyses. Mean levels of TTX and 6-epi-TTX for populations that possessed these compounds were compared by one-way ANOVA. Post-hoc pairwise comparisons of all population means were done by using the Student-Newman-Keuls test.

RESULTS

We found TTX in newts from four of seven sites (Table 1, Figure 3). Animals from Oregon (Tenmile, Benton County) and Washington (Grays Harbor, Beaver Lake) possessed TTX, while no individual from either Vancouver Island (Qualicum) or islands adjacent to Vancouver Island (Reid Island, Texada Island) possessed TTX (Table 1, Figure 3). In both populations from Oregon and one population from Washington (Grays Harbor), all of the individuals had TTX. In the second population from Washington (Beaver Lake) only two of four individuals had TTX.

Significant differences in TTX levels were found among newt populations with TTX ($F = 28.889$, $df = 3$, $P = 0.0001$). Pairwise comparisons indicated that significant differences existed between all population pairs, with the exception

TABLE 1. COMPARISON OF LEVELS OF TTX AND 6-epi-TTX OF 7 POPULATIONS OF *Taricha granulosa* FROM THE PACIFIC NORTHWEST

Population	Sample size (N)	TTX (mg/g skin, mean ± SE)	6-epi-TTX (mg/g skin, mean ± SE)
Benton County, OR	5	1.024 ± 0.142	0
Tenmile, OR	5	1.752 ± 0.110	0.236 ± 0.027
Grays Harbor, WA	4	0.143 ± 0.031	0.271 ± 0.125
Beaver Lake, WA	4	0.007 ± 0.004	0
Qualicum, BC	1	0	0
Texada Island, BC	3	0	0
Reid Island, BC	4	0	0

of Beaver Lake–Grays Harbor (Table 2). Mean levels of TTX were highest in populations from Oregon and lower in Washington (Tables 1 and 2). Because 220 ng of TTX kills one mouse [ddy, male, 15–20 g body weight (Fuhrman, 1986)], on average 1 g of skin from individuals from Oregon contained enough TTX to kill between 5000 (Benton County) and 9000 (Tenmile) mice, while 1 g of skin from individuals from Grays Harbor had enough TTX to kill 650 mice. Beaver Lake, the mainland population closest to Vancouver Island, had the lowest mean level of TTX (Tables 1 and 2), but 1 g of skin of an average individual from this population still had enough TTX to kill 32 mice. We found at least some intrapopulational variation in TTX levels in all populations from Oregon and Washington (Figure 4).

Newts from only two populations (Tenmile and Grays Harbor) possessed the C-6 stereoisomer of TTX, 6-epi-TTX (Table 1, Figure 3), and there was no significant difference between the mean levels of 6-epi-TTX of these populations ($F = 0.277$, $df = 1$, $P = 0.61$). In these samples, all individuals possessed 6-epi-TTX. No newts from either Benton County or Beaver Lake had 6-epi-TTX (Figure 3). All newts from Benton County and Tenmile possessed 4-epi-TTX (Figure 3), but concentrations of this analog were too low to quantify accurately. Other TTX analogs, including 4,9-anhydro-TTX and 4,9-anhydro-6-epi-TTX, were present in all individuals from populations in Oregon and Washington (Figure 3), but these compounds are unlikely to be important in the antipredator ecology of *Taricha* because of their low toxicity relative to TTX and 6-epi-TTX (Yasumoto and Yotsu-Yamashita, 1996). These anhydro isomers of TTX (i.e., 4,9-anhydro-TTX and 4,9-anhydro-6-epi-TTX) have been shown to have one-fiftieth the binding affinity of TTX in frog skeletal muscle and squid axon Na^{+} channels (Kao and Walker, 1982; Kao and Yasumoto, 1985; Yang et al., 1992). Although relative toxicity of these anhydro analogs in snakes is unknown, these analogs are unlikely to possess dramatically different toxicity.

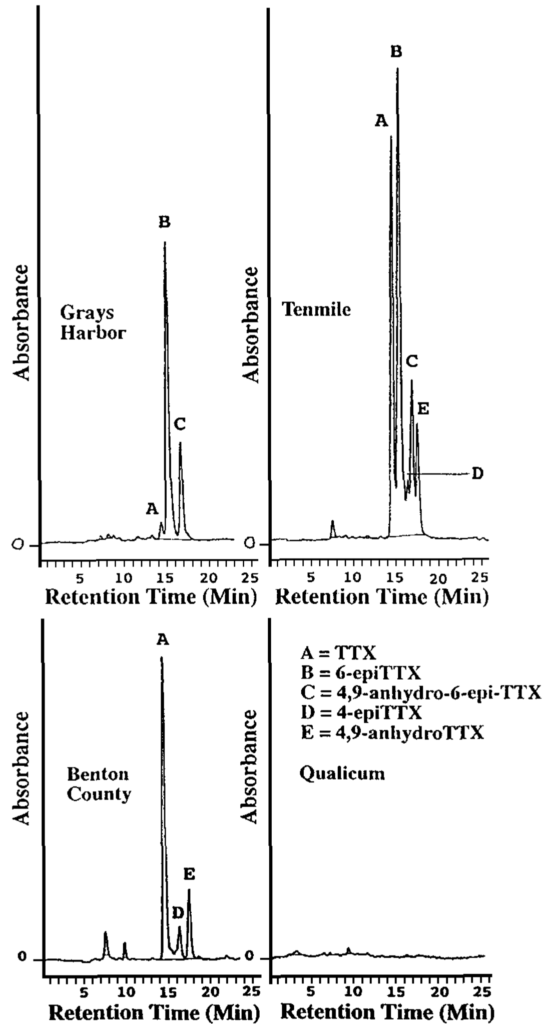

FIG. 3. HPLC chromatograms of representative individuals from four different populations showing differences in the amount of TTX present as well as differences in the types of analogs present. Newts from Qualicum, Vancouver Island, British Columbia, lack TTX and TTX analogs. Newts from Grays Harbor, Washington, and Tenmile, Oregon, show the presence of 6-epi-TTX, which is lacking in newts from Benton County, Oregon. Peak area for each compound is linearly related to its quantity. It is important to note that our analyzer is 20 times more sensitive to 6-epi-TTX than to TTX; thus, direct comparison of TTX and 6-epi-TTX peak area is not an accurate method of gauging the relative abundance of those compounds.

TABLE 2. PAIRWISE POST-HOC COMPARISONS OF MEAN TTX LEVELS FOR ALL POPULATIONS[a]

Population	Compared with	Critical difference $\sqrt{(\text{TTX ng/g skin})}$		Observed Difference $\sqrt{(\text{TTX ng/g skin})}$
Beaver Lake	Grays Harbor	7.06	n.s.	5.5
	Benton County	8.62	[b]	19.6
	Tenmile	9.58	[b]	27.2
Grays Harbor	Benton County	7.06	[b]	14.0
	Tenmile	8.62	[b]	21.6
Benton County	Tenmile	7.06	[b]	7.6

[a]Student-Newman-Keuls test was used. Tests were performed on square root transformations of ng/g measurements.
[b]Significance levels of $P < 0.05$.

FIG. 4. Graph of individual values (mg TTX/g skin) of tetrodotoxin for all individuals from populations where TTX was present. Both interpopulation and intrapopulation variation is present, although small sample sizes make precise evaluation of intrapopulation variation in TTX levels difficult.

DISCUSSION

Quantifying the variation in TTX levels of *Taricha granulosa* is a requisite step in evaluating whether coevolution occurs between the newt and its garter snake predator. Previous studies of this system have focused on variation within and among populations of the predator (Brodie and Brodie, 1990, 1991). The results presented here document a pattern of variation within and among newt

populations that is both necessary for, and consistent with, the occurrence of chemically mediated coevolution between predator and prey.

If TTX is the central trait in a coevolutionary interaction between *Taricha granulosa* and *Thamnophis sirtalis*, then we should see a pattern of matched exploitative ability and defense among populations of the two species (Brodie and Brodie, 1991, 1999; Thompson, 1994; Berenbaum and Zangerl, 1998). Our analysis of TTX levels confirms an explicit link between the chemical ecology of prey and predator species and reveals some unexpected patterns of geographic variation. Newts from Vancouver Island (and surrounding islands) possess no TTX and are sympatric with snakes that lack TTX resistance (Brodie and Brodie, 1991). Where snakes are known to be highly resistant to TTX (Brodie and Brodie, 1990), newts possess high levels of TTX (Benton County, Tenmile). Geographically intermediate (Washington) populations of newts have low, but highly variable, levels of TTX.

Dramatic population differences in TTX levels of newts may occur on a fine geographic scale. Populations at Benton County and Tenmile are separated by about 70 km, yet significant differences existed in mean TTX levels in these two populations. In addition, these populations differ substantially in the presence of some TTX analogs. Snakes at these two sites differ slightly in their average resistance to TTX as well (Brodie and Brodie, 1999). Newts and snakes from Tenmile exhibit somewhat higher levels of TTX and TTX resistance, respectively. The pattern of geographic variation in both defensive and exploitative abilities is consistent with the geographic mosaic perspective of coevolution that predicts coevolution will occur at the population scale and may result in different outcomes in different localities (Thompson, 1994).

Individual, heritable variation in trait values must be present at both sides of the phenotypic interface for coevolution to occur within any single population. These criteria have been demonstrated for TTX resistance in *Thamnophis sirtalis* (Brodie and Brodie, 1990), but have not been previously explored in *Taricha granulosa*. While our experimental design does not allow us to speculate on the inheritance of TTX levels in newts, we do observe individual differences in TTX levels that selection might act upon. Individual variation ranged from quantitative differences in TTX concentrations within most populations, to discrete differences in the population at Beaver Lake, where two of the four individuals sampled completely lacked TTX and TTX analogs.

Conclusions about the interpopulation and intrapopulation variability of newt TTX levels are limited by the small sample sizes of our study. Additional sampling at one or more populations may change quantitative estimates of mean toxicity or variation within some populations. However, significant population differences in toxicity do exist. Additionally, our estimate of mean TTX levels of newts from the Benton County population closely matched previously published estimates (Brodie et al., 1974). We view our data as preliminary evidence

supporting a rough pattern of coevolution between these two species rather than a definitive assessment of patterns of geographic variation in the TTX levels of *Taricha granulosa.*

The genetic control of TTX production in taxa that possess the toxin is poorly understood. Tetrodotoxin in pufferfish is thought to originate from bacteria (Yotsu et al., 1987; Yasumoto and Yotsu-Yamashita, 1996, but see Matsumura, 1995), but this condition has not been shown in newts. Toads of the genus *Atelopus* possess TTX in the wild, but do not produce TTX when raised in captivity (Daly et al., 1997) indicating that at least some aspect of their TTX production is affected by environmental factors. The existence of newts that either possess TTX or lack TTX from a single population (Beaver Lake) may indicate that TTX production is not entirely environmentally determined. If newts are producing their own TTX, a number of factors, including diet, ontogeny, and season, are likely to affect the toxicity of individual newts (Shimizu and Kobayashi, 1983; Daly et al., 1997).

Finally, our results suggest that 6-epi-TTX is the only TTX analog of possible ecological importance in interactions between newts and snakes. While we found multiple TTX analogs in our populations (including 6-epi-TTX, 4,9-anhydro-6-epi-TTX, 4-epi-TTX, and 4,9-anhydro-TTX), only 6-epi-TTX and 4-epi-TTX are likely to be sufficiently toxic (Yasumoto and Yotsu-Yamashita, 1996) to be biologically significant. Although data on the relative toxicity of TTX analogs are based on data from squid axon and frog skeletal muscle (Kao and Walker, 1982; Kao and Yasumoto, 1985; Yang et al., 1992), the action of TTX and the structure of the TTX binding site in Na^+ channels makes it unlikely that either 4,9-anhydro-6-epi-TTX or 4,9-anhydro-TTX possess much toxicity in garter snakes. Of the analogs with high toxicity (6-epi-TTX and 4-epi-TTX), only 6-epi-TTX was present at concentrations that are likely to generate toxic effects. On average, individual newts from both Tenmile and Grays Harbor possessed enough 6-epi-TTX to kill 500 mice, indicating that it could play an important role in the antipredator arsenal of newts from these populations. As with TTX, interpopulational variation in the presence or absence of 6-epi-TTX was detected, but no variation was seen in the mean levels between 6-epi-TTX-bearing populations. Why newts from some populations possess other TTX analogs but do not possess 6-epi-TTX remains to be explained, as does the homogeneity of mean 6-epi-TTX levels in populations where it is present. Answers to questions about TTX analog variability may shed light not only on the coevolutionary interaction between *Taricha granulosa* and *Thamnophis sirtalis* but also on the biosynthesis of TTX.

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INDUCTION OF A *p*-COUMAROYL TRIHYDROXY TRITERPENE ACID IN *Psylla*-INFESTED AND MECHANICALLY DAMAGED PEAR TREES

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Abstract—The pattern of induction and the chemical structure of phenolic compounds in pear trees (*Pyrus communis*, cv. Conference) that were either infested by pear leaf suckers *Psylla pyricola* and *P. pyri* or mechanically damaged, or both, were studied. Chromatographic (HPLC) and mass spectral analysis performed on extracts of leaf samples collected at various time intervals from trees subjected to three treatments demonstrated the induction (and/or amplification) of a phenolic compound, identified as 3-*O*-*trans*-*p*-coumaroyltormentic acid (I). New mass spectrometric data on this phenolic compound are presented. HPLC revealed different peak patterns in the course of the period of *Psylla* infestation and the lapse of time since mechanical damage was inflicted, compared to a control tree. The new phenolic compound became apparent after 12 hr and reached the highest level 30 days after damage by pear leaf suckers. It was also observed after 24 hr at lower intensity in samples from a mechanically damaged tree and exclusively on day 30 at very low intensity in the leaf extracts from the uninfested control trees. We conclude that damage by pear leaf suckers, and to a lesser extent also mechanical damage, induce the synthesis of the new, late-eluting phenolic compound. We propose that this compound is involved in plant defense against pear leaf suckers.

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Key Words—*Pyrus communis* cv. Conference; Rosaceae, pear, *Psylla pyricola*, leaf sucker, phenols, induced plant defense, 3-*O*-*trans*-*p*-coumaroyl-tormentic acid, mass spectrometry.

INTRODUCTION

Natural or mechanical damage can change the secondary metabolism of a plant and provide the means to fight the changing circumstances in that plant by increasing its natural resistance (Bernays, 1994). The detection and identification of products (secondary metabolites) involved in the processes that take place during the damage period can give useful information on the direct defense system in a plant.

Phenolics are a large group of secondary plant metabolites that include, among others, compounds derived from the condensation of acetate units (e.g., terpenoids) or by modification of aromatic amino acids (e.g., phenylpropanoids, cinnamic acids, coumarins, and others). Constitutive phenolics can act as insect feeding deterrents, inhibitors of digestive enzymes, and resistance against fungi and bacteria (Kodoma et al., 1998). Their concentrations may vary considerably among individual plants (Scutareanu and Lingeman, 1992), but they may increase due to de novo synthesis after herbivory or mechanical damage (Summers et al., 1994).

Various studies on the occurrence of phenolic compounds in pear trees are available in the literature (Chalisse and Williams, 1968; Chalisse and Westwood, 1972; Hegnauer, 1973; Chalisse et al., 1980; Matias et al., 1990; Gerard et al., 1993). To our knowledge, chemical characterization of specific phenolics synthesized in wounded or naturally damaged pear trees has not been performed previously.

In a preliminary experiment we showed that a group of unidentified compounds with late retention time and hydrophobic behavior was dominant upon reversed-phase HPLC of acetone extracts of young leaves from pear trees of the variety Conference infested by *Psylla pyricola* Foerster (= pear leaf sucker), but the compounds were virtually absent (the relative abundance is extremely low (<1%) compared to other compounds detected at 280 nm) from uninfested young and uninfested and infested mature leaves (Scutareanu et al., 1996a). In addition, we reported normalized HPLC chromatograms from a series of pear leaf samples taken in the course of a *Psylla*-infestation period, from mechanically wounded pear leaves, and from appropriate controls (Scutareanu et al., 1996b). In this article, we present the chemical structure elucidation of the phenolic compound induced in microgram quantities by *Psylla* infestation on leaves and/or mechanical wounding of 1-year-old Conference pear trees, and we assess how its production changes in the course of a *Psylla* infestation period as compared to mechanically wounded and uninfested trees.

METHODS AND MATERIALS

Plants

Four 1-year-old pear trees (*Pyrus communis* L. cv. Conference), grafted on pear rootstock Kwee C, were obtained from the commercial nursery Boomkwekerijen C. van Diepen B.V., Biddinghuizen, Flevoland, The Netherlands.

Insects

Psylla pyricola Foerst. and *P. pyri* L. (Homoptera, Psyllidae) (= pear leaf suckers), were collected from a natural population located in a pear orchard in Watergraafsmeer (Amsterdam, The Netherlands).

Experimental Set-Up, Location, and Treatments

Four potted pear trees were placed, 2 × 2, in separate cages in a climate room (±23°C; 65–70% relative humidity; 16L : 8D). Two trees in the first cage gradually became infested after the release of *P. pyricola* and *P. pyri* adults, but one tree was mechanically damaged by cutting twigs in half with an unsterilized scalpel. In the second cage, the trees were kept uninfested (free of any insect infestation), but one tree was mechanically damaged as in the first cage.

Sampling Procedure

Five to ten leaves were sampled randomly from each tree, before any infestation or damage had taken place (T0), after *Psylla* nymphs appeared on leaves (and/or mechanical damage was inflicted) (T1), 12 (T2), 24 hr (T3), and 2 (T4), 5 (T5), 14 (T6), and 30 days (T7) later.

Assessment of Infestation Level

Each leaf sample was inspected under a binocular microscope, and all stages of psyllids were counted, but only the number of nymphs are presented in Figure 1 to characterize the degree of infestation.

Analysis of Polyphenols

Solid/Liquid-Phase Extraction. All psyllid stages, excretions (honeydew), and feces were removed, and the fresh sampled pear leaves were rinsed in acetone (15 ml/g) to dissolve the epicuticular tissue. After filtration, the dried extract was dissolved in 250–500 μl ethanol/g fresh leaf and stored at −20°C. Before HPLC analysis, each extract was centrifuged and diluted 10× in ethanol. For collection of fractions for MS and NMR, the extract was dissolved in 2 ml 80% methanol. After centrifugation, the supernatant solution was applied on a C_{18}

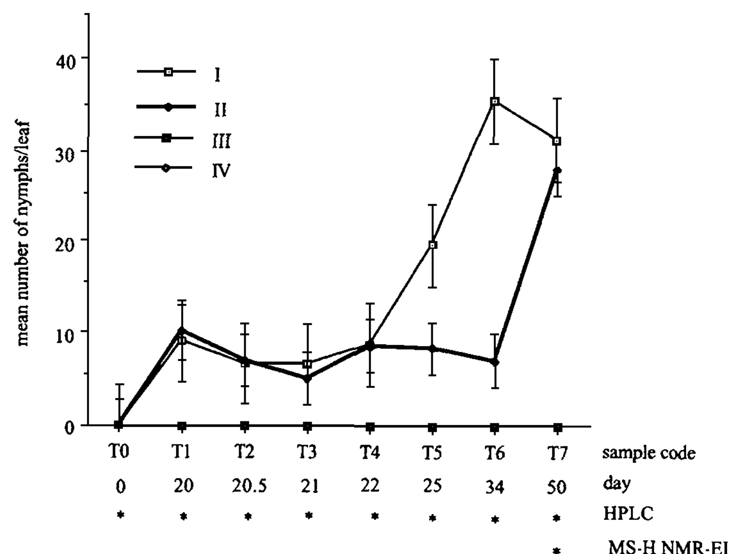


FIG. 1. The abundance (mean number ± SE) of *Psylla* nymphs on leaves of pear trees under the treatments: I = *Psylla*-infested; II = *Psylla*-infested and mechanically damaged; III = uninfested, undamaged; IV = mechanically damaged. Day 0 = before infestation and damage; day 20 = first nymphs on leaves and infliction of mechanical damage; day 20.5 = after 12 hr; day 21 = after 24 hr; day 22 = after 2 days; day 25 = after 5 days; day 34 = after 14 days; day 50 = after 30 days. *Refers to performed chemical analyses.

cartridge (200 mg solvent, Varian) to remove lipophilic pigments, such as chlorophyll. The C_{18} cartridge was pretreated by washing with 3 ml methanol and 3 ml distilled water. Elution was performed with 3 ml 80% methanol.

HPLC Analysis. The chromatograms from leaf extracts obtained on each of the eight sampling times (T0–T7) were obtained from LDC-Milton Roy CM-4000 equipment, encompassing a Waters 996TM solvent delivery system (Waters, Milford, Massachusetts). The HPLC conditions were as follows: C_{18} column; solvent A, 95% water + 5% acetonitrile; solvent, B, 25% water + 75% acetonitrile + phosphoric acid (pH 2) used to acidify the eluent; injection volume, 20 μl; flow rate, 0.8 ml/min; detection, 280 nm; and running time, 60 min. The data and the chromatographic process were controlled by a MACRO 6 computer. To normalize the original chromatograms, the intensity of the peaks was expressed as a percentage of the sum of all peak heights.

HPLC analyses were repeated with a Waters 600MS solvent delivery system, a Rheodyne valve with an injection volume of 100 μl (Achrom, Cotati, California), and a Waters 991 photodiode array (PDA) detector. The chromatographic and data processing were controlled with Millenium software (Waters).

The mobile phase was a two-solvent system consisting of solvent A (1% formic acid in Milli-Q water) and solvent B (1% formic acid in HPLC gradient grade methanol). Solvents were filtered through a Nylon 66 membrane and degassed by helium purging. A gradient profile was applied as follows: 0–25 min linear gradient 10–50% B, 25–30 min isocratic 50% B, 30–50 linear 50–80% B, 50–53 min linear gradient 80–100% B, and 53–73 min isocratic 100% B. The flow rate was 1 ml/min. Detection was at 280 and 320 nm, and the on-line UV spectra were recorded between 220 and 400 nm.

Spectroscopic Analysis: The ^1H NMR spectrum was recorded in CD_3OD on a Varian Unity 400 spectrometer, operating at 400 MHz at room temperature. Chemical shifts are reported in parts per million (δ) downfield from tetramethysilane (TMS, 0 ppm). Electrospray (ES) mass spectra were obtained with a VG Quattro-II tandem quadrupole mass spectrometer (Fisons, VG Biotech, Manchester, UK) in both positive and negative ion modes. Product ion spectra were measured by collisional activation with argon in the hexapole gas cell at a collision energy of 15 eV. The fast atom bombardment (FAB) and low- and high-resolution EI spectra were obtained on a VG 70 SEQ (Micromass, Manchester, UK) hybrid mass spectrometer. Product ion spectra of selected fragment ions were obtained by high-energy (8 keV) collision-induced dissociation with helium as target gas and employing 50% beam attenuation in the first field-free region and B/E linked scaning.

The structures of compounds I and Ia are shown in Figure 2a and mass spectral and NMR data are as follows: (*R*)-3-β-*trans-p*-coumaroyloxy-2a,19a-dihydroxyurs-12-en-28-oic acid (I, trivial name: 3-*O-trans-p*-coumaroyltormentic acid): colorless; UV λ_{max} (on-line): 312 nm (MeOH-H_2O-HCOOH). Electrospray-MS *m/z*: 657 $[M + Na]^+$, 635 $[M + H]^+$, 633 $[M - H]^-$ and 316 $[M - 2H]^2$; FAB-MS (matrix: glycerol) *m/z*: 657 $[M + Na]^+$, 635 $[M + H]^+$, and 633 $[M - H]^-$; ^1H NMR (400 Hz, CD3OD) δ ppm: 0.85, 0.94, 0.96, 1.07, 1.20 and 1.35 (each 3H, s), 0.93 (3H, d, J = 7.2 Hz, 20-Me), 2.59 (1H, s, 18-H), 3.57 (1H, m, 2β-H), 4.63 (1H, d, J = 9.9 Hz, 3α-H), 5.30 (1H, brs, 12-H), 6.38 and 7.63 (each 1H, d, J = 15.8 Hz), 7.63 and 6.81 (each 2H, J = 8.5 Hz).

Methyl 3-β-*trans-p-O*-methylcoumaroyloxy-2α,19α-dihydroxyurs-12-en-28-oate (Ia): The dimethyl derivative was prepared with ethereal diazomethane. EI-MS *m/z* (rel. int.): 662 (M^+, 0.3), 602 (0.5), 484 (13), 424 (53), 352 (16), 260 (12), 219 (27), 218 (25), 206 (26), 205 (29), 201 (38), 179 (100), 161 (22) and 146 (65); High-resolution EI-MS: 662.4119 (M^+) (calcd. for $C_{41}H_{58}O_7$: 662.4183), 484.3559 (calcd. for $C_{31}H_{48}O_4$ 484.3553), 424.3326 (calcd. for $C_{29}H_{44}O_2$: 424.3341), 352.2723 (calcd. for $C_{25}H_{36}O_1$: 352.2766), 260.1810 (calcd. for $C_{17}H_{24}O_2$: 260.1776), 219.1749 (calcd. for $C_{15}H_{23}O_1$: 219.1749), 218.1674 (calcd. for $C_{15}H_{22}O_1$: 218.1670), 206.1660 (calcd. for $C_{14}H_{22}O_1$: 206.1671), 205.1604 (calcd. for $C_{14}H_{21}O_1$: 205.1592), 201.1637 (calcd. for $C_{15}H_{21}$: 201.1643), 200.1558 (calcd. for $C_{15}H_{20}$: 200.1565), 179.1052 (calcd.

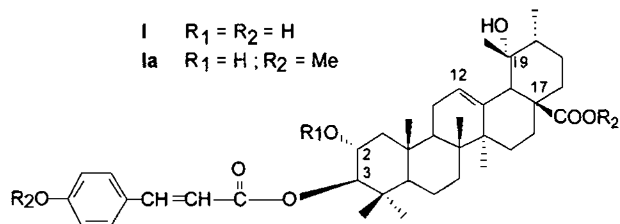


FIG. 2. Structure of 3-*O*·*p*-coumaroyltormentic acid (I) and its dimethyl derivatives (Ia).

for $C_{11}H_{15}O_2$: 179.1072), 161.0627 (calcd. for $C_{10}H_9O_2$: 161.0603), 146.1092 (calcd. for $C_{11}H_{14}$: 146.1096).

RESULTS

HPLC Patterns Related to Natural (Psyllids) and/or Mechanical Damage

HPLC analysis performed on extracts of fresh young leaves of 1-year-old pear trees revealed different peak patterns. For all leaf samples taken before the trees incurred any damage, two major peak groups eluting before 25 min (1500 sec) were observed. Tentative indentification indicated that they correspond to known compounds previously reported in undamaged pear trees (see Introduction section and Figure 3 legend). However, in the analysis of leaf extracts from samples collected from *Psylla*-infested (natural) and/or mechanically damaged trees, a new late-eluting peak (peak 30, Figure 3b, d, and f) with a retention time between 41 and 51 min appeared in the chromatograms. This peak was virtually absent from all the samples collected from the same trees before infestation and/or damage (Figure 3a, c, and e) and from an uninfested/undamaged control tree.

There was a close relationship with progress of infestation and the elapse of time since the mechanical damage was inflicted. The relative abundances of peak 30 in extracts from leaf samples collected from treated and control trees are summarized in Table 1. This peak was noted within 12 hr (T2) after the appearance of the nymphs on leaves and, with a lower intensity, after 24 hr (T3) in samples from the mechanically damaged tree. Its highest intensity was reached a day 30 (T7) after the appearance of the nymphs on leaves from the *Psylla*-infested tree. In the pear tree subjected only to mechanical damage, peak 30 also showed high intensity at day 30 but, in terms of relative abundance, was lower than at day 14. In the pear tree subjected to both natural and mechanical damage (not shown in Figure 3), the late-eluting peak appeared after 12 hr and reached an intermediate level of intensity at day 30, while in the extract of the

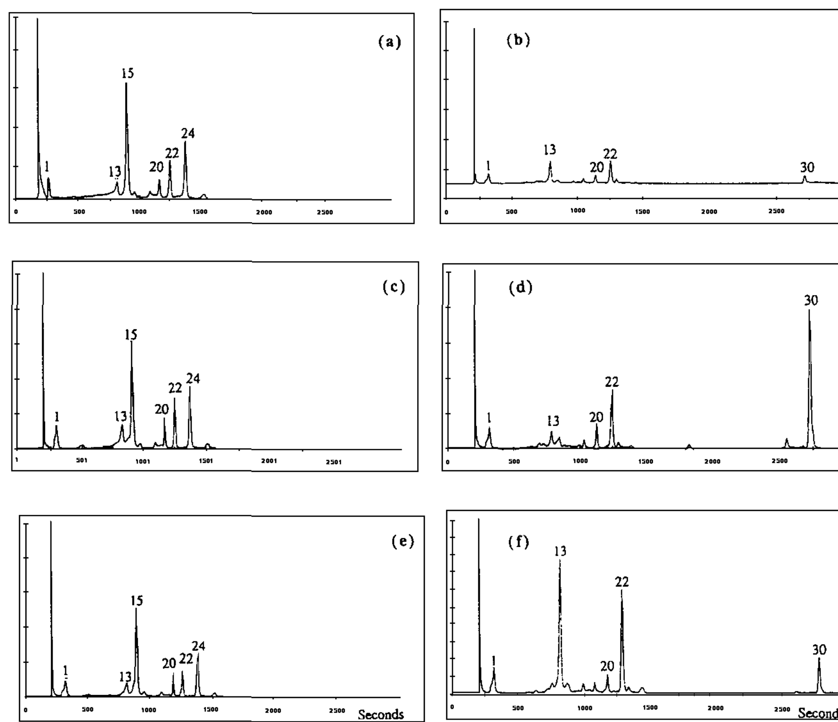


FIG. 3. Characteristic HPLC chromatograms obtained from acetone extracts of pear tree leaves. Treatments a and b: uninfested, undamaged; c and d: *Psylla*-infested; e and f: mechanically damaged; a, c, e: patterns before infestation and damage; b, d, f: patterns 30 days after appearance of first nymphs on leaves or mechanical damage were inflicted. UV detection wavelength was 280 nm. Numbers were given only to the peaks holding at least 1by cochromatography with available reference compounds and UV properties: 1 = arbutin; 15 = chlorogenic acid; 24 = dicaffeoylquinic acid.

last leaf sample from the uninfested/undamaged control tree this peak revealed a very low intensity only after 30 days (Figure 3b). Hence, we concluded that both natural and mechanical damage amplify the accumulation of a phenolic compound, which could be involved in the resistance or defense of the pear trees cv. Conference against per leaf suckers.

Structural Elucidation of 3-O-trans-p-Coumaroyltormentic Acid

In the repeated HPLC analysis with PDA detection and a methanol–water (1% formic acid) solvent system, peak 30 (Figure 3d and f), induced by damage, could be resolved into components (ratio 1 : 4) showing the characteristic UV

TABLE 1. RELATIVE ABUNDANCES (PROPORTION) OF LATE-ELUTING PEAK 30[a]

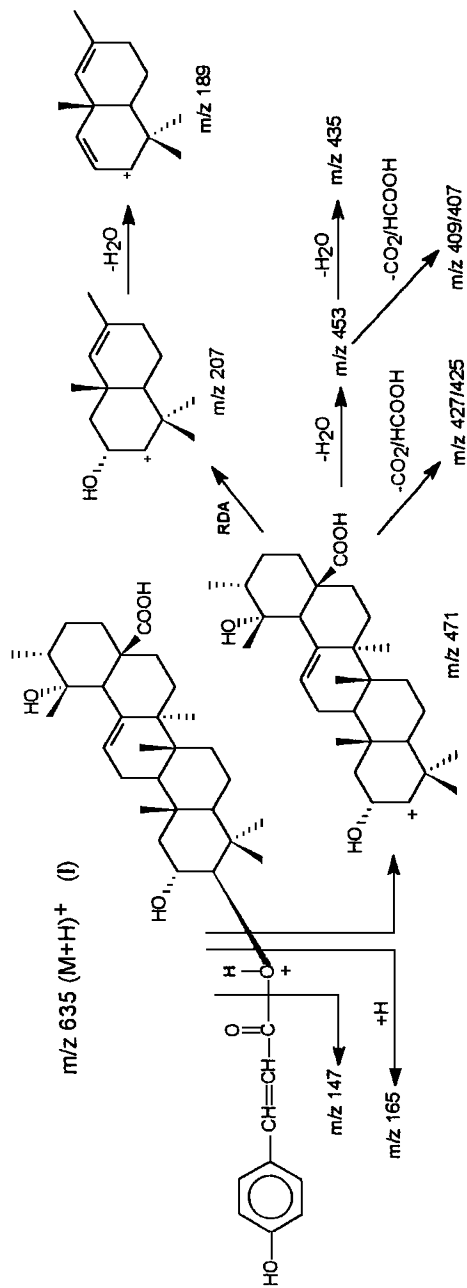
Sample code	Uninfested/ undamaged	*Psylla*-infested	Mechanically damaged	*Psylla*-infested and mechanically damaged
T0	0	0	0	0
T1	0	0	0	0
T2	0	0.015	0	0.06
T3	0	0.03	0.02	0.02
T4	0	0.02	0.01	0.015
T5	0	0.09	0.07	0.03
T6	0	0.26	0.115	0.03
T7	0.07	0.43	0.085	0.08

[a]Determined from the total peak heights in chromatograms as a function of the time elapsed from the appearance of nymphs on the leaves and the infliction of mechanical damage in treatment and control pear trees cv. Conference. T0 = before infestation and damage; T1 = appearance of nymphs and mechanical damage; T2 = after 12 hr; T3 = after 24 hr; T4 = after 2 days; T5 = after 5 days; T6 = after 14 days; T7 = after 30 days.

absorption of *cis*- and *trans*-coumaroyl groups with the same molecular weight (634), consistent with the occurrence of *cis* and *trans* isomers. The detailed structural elucidation has only been performed for the major isomer (*trans* isomer).

The positive electrospray and FAB mass spectra of the late-eluting compound (I) showed a protonated $[M + H]^+$ and a sodiated [M + Na] molecule at m/z 635 and 657, respectively, whereas the negative electrospray and FAB spectra revealed a deprotonated molecule $[M - H]^-$ at m/z 316, suggesting two acidic groups. Further product ion analysis of the $[M + H]^+$ ion (m/z 635) provided support for the presence of a coumaroyloxyl group (m/z 165, 147, and 471), two free OH functions (m/z 453 and 435 from m/z 471) and a —COOH group (m/z 427/425 and 409/407 from m/z 471) (Scheme 1).

After methylation with diazomethane, this acid (I) afforded a dimethyl derivative containing a 4′-*O*-methylcoumaroyl group (Ia), which was found to correspond to $C_{41}H_{58}O_7$ by accurate mass measurement in the high-resolution EI mode. Major fragment ions obtained in the EI mass spectrum of Ia appear at m/z values of 484, 424, 352, 219, 218, 206, 205, 201, 179, and 146. The product ion spectrum of the fragment ion at m/z 484, obtained by the loss of 4′-*O*-methylcoumaric acid (loss of 178) from the $M^{+\cdot}$ ion (m/z 662), provided useful structural information (Figure 4, Scheme 2). The product ions at m/z 278 and 206/205 formed by a retro-Diels-Alder (RDA) fragmentation in ring C and indicated a methyl Δ^{12}-unsaturated triterpene-28-oate with a hydroxyl group in ring D or E, and the other hydroxyl group in ring A or B (Djerassi et al., 1962; Budziekiewicz et al., 1963). Additional product analysis performed on the major fragment ion m/z 424 observed for the methylate (Ia) upon EI analysis indi-



SCHEME 1. Fragmentation of protonated I (*m/z* 635) generated by electrospray ionization upon low-energy collision-induced dissociation.

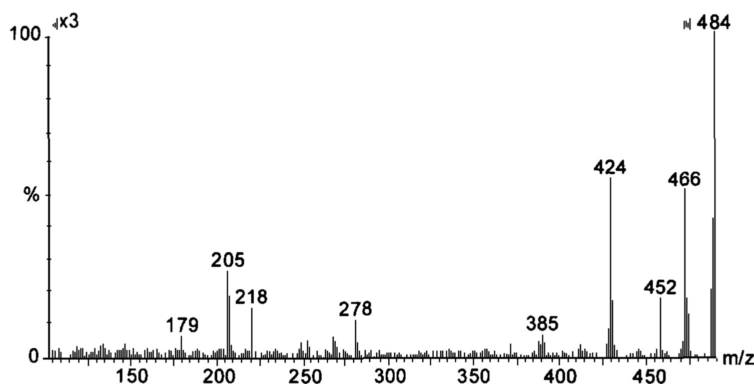
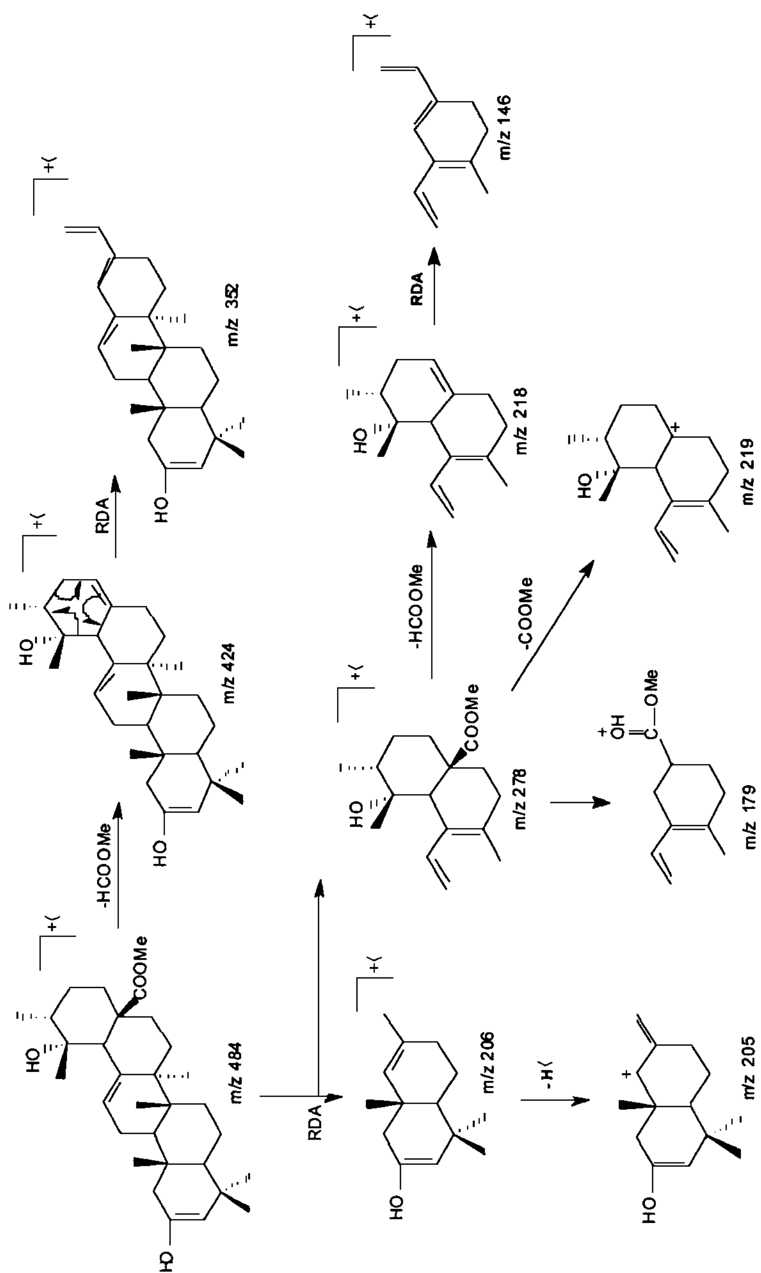


FIG. 4. B/E linked scan product ion spectrum of the *m/z* 484 ion obtained by EI of Ia.

cated further loss of 72 u, yielding a highly stabilized radical cation at *m/z* 352 (Scheme 2). The latter loss could be explained by a RDA fragmentation in the E ring, strongly supporting the presence of a carbomethoxyl group at C-17 and confining the hydroxyl group to the E ring. RDA fragmentation in the E ring also allows us to rationalize the formation of the *m/z* 146 ion from the *m/z* 278 ion. The assignment of all product ions in Scheme 2 (except *m/z* 278, which was too weak) was further confirmed by accurate mass measurements employing high resolution EI-MS.

In addition, the *m/z* 179 ion, which corresponded to the base peak in the EI spectrum of Ia and related triterpenes containing the structure methyl 19α-hydroxyurs-12-en-28-oate (Ojinnaka et al., 1980; Ogura et al., 1977a), could be rationalized by a McLafferty-type rearrangement involving δ-hydrogen transfer and subsequent radical-induced C—C cleavages. This ion appeared to be diagnostic of methyl 19-hydroxyurs-12-en-28-oate because it could not be detected in the EI spectra of similar triterpenes without a 19-OH group (Ogura et al., 1997a; Yagi et al., 1978).

The ^{1}H NMR spectrum (CD_3OD) of the original compound (I) clearly showed six tertiary methyls, a secondary methyl (0.93 ppm, d; J = 7.2 Hz), and two AB type signals (7.63 and 6.38 ppm, each 1H, J = 15.8 Hz; 7.47 and 6.81 ppm, each 2H, J = 8.5 Hz), indicating a *trans-p*-coumaroyl group. The chemical shift and the singlet nature of the signal due to H_{18} (2.59 ppm, 1H, s) revealed that the tertiary hydroxyl group is located at C-19 and has an α-orientation (Takahashi and Takani, 1978; Ojinnaka et al., 1980). This was also supported by the deshielding of the C-27 methyl signal (1.35 ppm, 3H, s) (Ogura et al., 1977b). The chemical shifts and the J value for a doublet signal at 4.63 ppm (1H, d, J = 10 Hz, $H_{3\alpha}O$ (Kojima and Ogura, 1989), due to a proton on a carbon bearing an ester oxygen atom, and comparison with published data for



SCHEME 2. Major fragmentations of the dimethyl derivative 1a in EI-MS and confirmed by product ion analyses and high-resolution measurements.

related hydroxycinnamic acid derivatives (Ogura et al., 1977a; Yagi et al., 1978; Numata et al., 1989; Sashida et al., 1994; Hamberlein and Tschiersch, 1994) of triterpene acids indicated the presence of a 2α-OH,3β-coumaroyloxyl configuration. Additional evidence (UV_{max} = 312 nm) also eliminated the alternative possibility of a 2α-coumaroyloxyl, 3β-OH configuration (Siddiqui et al., 1987).

On the basis of the above spectroscopic data, the original compound (I) was assigned as 3-β-*trans-p*-coumaroyloxy-2α,19α-dihydroxyurs-12-en-28-oic acid. This compound was previously isolated from a Chinese traditional medicine, Goreishi (the feces of *Trogopterus xanthipes* Milne-Edwards, the complex-toothed flying squirrel) and was named 3-*O-trans-p*-coumaroyltormentic acid (Numata et al., 1989).

DISCUSSION

The increased production of a phenolic compound of high molecular weight (634) with hydrophobic properties detected by reversed-phase HPLC was demonstrated in both *Psylla*-infested and mechanically damaged pear trees. Structural elucidation showed that in both types of damage the major induced phenolic compound is 3-*O-trans-p*-coumaroyl-2α-19α-trihydroxyurs-12-en-28-oic acid. However, the intensity differed greatly between mechanically damaged and *Psylla*-infested trees. Mechanical injury is known to have variable effects and, in any case, induces production of phenolics to a smaller extent than insect injury (Neuvonen and Haukioja, 1984; Haukioja and Hanhimaki, 1985; Kogan and Fischer, 1991; Hartley and Lawton, 1991). The induced localized or systemic responses occurring upon damage become manifest within hours after *Psylla* infestation or days after mechanical injury and is most manifest in young leaves (Scutareanu et al., 1996a). Whether there are also induced responses in pear trees at a longer time scale, i.e., the next season's foliage or even later (Karban and Baldwin, 1997), remains to be investigated.

It should be mentioned that the induced response to mechanical damage may be triggered because we used unsterilized scissors for the treatment. Mechanical damage can cause different effects, depending on the kind of tool used for the damage. Cutting of leaves with sterilized scissors induced less accumulation of phenolics than cutting with scissors dipped in insect saliva (Hartley and Lawton, 1991). Whether the use of sterilized or nonsterilized scissors matters has not yet been investigated. We also should emphasize that our mechanical treatments did not imitate the process of leaf damage caused by psyllid adult and nymphs, which are exclusive phloem suckers.

It remains to be determined whether the phenolic compound identified in the present study is induced specifically by herbivory and mechanical damage alone. Plant responses often tend not to be tightly linked to specific stimuli.

For example, the accumulation of phenolic compounds in plants has been found to follow not only herbivory and mechanical leaf damage, but also pathogen infection, exposure to intense light, and nutrient limitation (Coleman and Jones, 1991).

Although each treatment involved just one tree, it should be emphasized that each tree was sampled eight times over a period of approximately seven weeks to study the dynamic changes in phenolics starting prior to any treatment. The results showed convincingly that the *p*-coumaroyl trihydroxy triterpene acid was absent from the first samples and only appeared after the treatments (*Psylla* infestation, mechanical damage, and the combination) in all six subsequent samples, whereas it was not present in the samples of the control tree except for the very last sample at a very low intensity. Therefore, we can be confident about the within-tree responses to the treatments. How specific the induced responses are is not yet clear. Specificity of elicitors should be distinguished from specificity of response. As advocated by Green and Ryan (1997), experiments involving a range of different treatments are required to obtain more insight into the specificity of the response (note that our study included four treatments). In addition, response specificity should be assessed by recording the effect of the induced phenolics on the performance of the psyllids. A preliminary study on the effect of pear trees treated with similar, commercially available phenolics showed decreased nymphal survival and oviposition performance of pear psyllids (Scutareanu, unpublished data).

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DO FOLIAR PHENOLICS PROVIDE PROTECTION TO *Heliothis virescens* FROM A BACULOVIRUS?

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Abstract—The effects of chlorogenic acid on larval survival and growth of the tobacco budworm *Heliothis virescens* and larval susceptibility to *Helicoverpa zea* nucleopolyhedrovirus (HzSNPV) were studied with an artificial diet and transgenic tobacco *Nicotiana tabacum*. Survival of neonates on over- or underexpressed phenylalanine ammonia-lyase (PAL) transgenic tobacco lines was positively correlated with the level of chlorogenic acid. Larval weight was not correlated with the level of chlorogenic acid in tobacco foliage. On the other hand, larval weights on artificial diet supplemented with chlorogenic acid were negatively correlated with chlorogenic acid concentration. Second instars treated on virus-treated (10 occlusion bodies/larva) tobacco leaf disks for 24 hr and then reared on tobacco leaves showed higher survival time and increased mortality on lines with increasing levels of chlorogenic acid. However, viral-related larval mortality on artificial diet was negatively correlated with dietary chlorogenic acid. These results illustrate the inadequacy of artificial diet studies in establishing causal relationships among plant chemistry, herbivores, and natural enemies.

Key Words—*Heliothis virescens*, chlorogenic acid, HzSNPV, baculovirus, phenolics, tritrophic interactions, plant defense.

INTRODUCTION

Abundant evidence suggests that host-plant chemistry influences the susceptibility of insect herbivores to disease (reviewed by Duffey et al., 1995; Hoover et al., 1998a–c; Ali et al., 1998). Phenolics (e.g., monomeric phenolics to polymeric tannins) have received the most notice due to their ubiquitous occur-

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rence, structural diversity, and broad antibiotic activity (Wink, 1988; Duffey et al., 1995; Hammerschmidt and Schultz, 1996). Several thousand phenolics have been characterized from plants (Koukol and Conn, 1961; Jones, 1984; Maher et al., 1994), and they have been critical in the development of plant–herbivore theories (Waterman and Mole, 1994).

A widely occurring plant phenolic is chlorogenic acid, a quinic acid ester of caffeic acid (Sondheimer, 1964; Harborne, 1979, 1982; Elliger et al., 1981; Isman and Duffey, 1982). Chlorogenic acid inhibits the growth of lepidopteran larvae when incorporated in artificial diets (e.g., Elliger et al., 1981; Felton et al., 1989; Stamp et al., 1994; Yang et al., 1996). Alternatively, chlorogenic acid and the flavonoid rutin significantly inhibited the infectivity of *Autographa californica* multiple nucleocapsid nucleopolyhedrovirus (AcMNPV) and *Helicoverpa zea* single nucleocapsid nucleopolyhedrovirus (HzSNPV) against *H. zea* (Felton et al., 1987). The oxidation of phenolics such as chlorogenic acid by foliar oxidative enzymes (e.g., peroxidases or polyphenol oxidases) also diminishes the infectivity of NPVs against *H. zea* and *Heliothis virescens* (F.) larvae (Felton and Duffey, 1990; Hoover et al., 1998a).

Other phenolics are also implicated as factors in mediating infectivity of insect diseases. Dietary tannins reduced the infectivity of *Lymantria dispar* nucleopolyhedrovirus (LdNPV) against *L. dispar* L. (Keating et al., 1988, 1989, 1990) and HzSNPV against *H. zea* (Young et al., 1995). Mortality of baculovirus-treated lepidopteran larvae fed on leaf disks of different tanniferous host plants varied significantly (Keating et al., 1988, 1990; Forschler et al., 1992).

Although there is considerable evidence that phenolics mediate susceptibility of insects to baculoviruses, previous investigations have relied upon correlational evidence or upon the use of artificial diets laced with the respective phenolic. More direct and incontrovertible evidence would be obtained by using transgenic plants where the content of specific phenolics varies. Phenylalanine ammonia-lyase (PAL) is a rate-limiting enzyme in phenolic biosynthesis (Bate et al., 1994). A bean *PAL2* gene was introduced into the tobacco genome, which led to transgenic lines overexpressing or underexpressing PAL-activity (Elkind et al., 1990; Howles et al., 1996; Pallas et al., 1996). These plants represent an ideal model system to investigate the biological effects of chlorogenic acid because it represents over 60% of the soluble phenolics in tobacco, and the levels of chlorogenic acid vary widely among the transgenic lines (Bate et al., 1994). We recently reported that, in contrast to studies based upon artificial diets (Stamp et al., 1994; Yang et al., 1996), *Manduca sexta* (L.) and *H. virescens* were largely unaffected by chlorogenic acid levels in transgenic tobacco leaves (Bi et al., 1997; Eichenseer et al., 1998).

The reasons for the apparent discrepancy between experiments with artificial diet and those with transgenic plants remain elusive. Previously, we showed that the toxicity of chlorogenic acid is dependent upon oxidation by phenolic oxi-

dases (Felton et al., 1989) or by autooxidation in the insect midgut (Summers and Felton, 1994). The diversity and quantity of antioxidants (e.g., tocopherols, carotenoids, glutathione, ascorbate, etc.) (Ahmad, 1992, 1995) found in plant tissues may attenuate phenolic toxicity. Artificial diets lack many of these antioxidants (e.g., carotenoids, glutathione). Since caterpillar growth on transgenic tobacco was unaffected by chlorogenic acid (Bi et al., 1997), the ability of phenolics to inhibit the infectivity of NPVs to lepidopteran larvae also warrants further examination.

Here, we use near isogenic PAL-transformed tobacco lines to assess the effects of phenolics on the susceptibility of *H. virescens* to HzSNPV. We report the impact of 18-fold variation in foliar chlorogenic acid content on the survival of *H. virescens* larvae in the presence and absence of the baculovirus.

METHODS AND MATERIALS

Plant and Insect Materials. To determine the effect of host-plant chlorogenic acid on the survivorship of *Heliothis virescens* larvae and their susceptibility to HzSNPV, 12 transgenic tobacco, *Nicotiana tabacum* cv. Xanthi-nc, lines were selected that have epigenetically suppressed or overexpressed levels of phenylalanine ammonia-lyase (PAL) and chlorogenic acid. The development of tobacco lines 274-T4, 160-P3, 104-T5 and 10-6T1 from *Nicotiana tabacum* cv. Xanthi-nc has been described (Elkind et al., 1990; Howles et al., 1996; Pallas et al., 1996). These lines contain the bean *PAL2* gene under the control of its own promoter as well as additional cauliflower mosaic virus 35S enhancer sequences. The 274-T4 and 160-P3 lines are sense-suppressed lines in which the introduction of the bean transgene reduced accumulation of tobacco PAL transcripts (Elkind et al., 1990). The line 104-T5 is a fifth generation line that partially recovered from the sense suppression and exhibits slightly decreased PAL activity (Bate et al., 1994). The line 10-6T1 changed from a sense-suppressed line to an overexpressing line in a single generation (Howles et al., 1996). Overall PAL activity in 10-6T1 is greater than the wild type, and the levels of endogenous PAL transcripts are returned to wild-type levels. The C-17 control lines lost the bean *PAL2* gene through segregation and are operationally wild type.

Plants were grown from stem cuttings of each tobacco line in 2-liter plastic pots filled with Redi-earth Peat-Lite Mix soil mixture (Scotts-Sierra Horticultural Products Company, Marysville, Ohio) in a greenhouse. Pots were arranged in a completely randomized experimental design. Plants were watered every day and fertilized with Osmocote (Scotts-Sierra Horticultural Products) (N-P-K = 14 : 14 : 20) monthly. Greenhouse conditions were 14-hr photophase, with high-pressure sodium light (100 W), and day–night temperature was 27 : 19 ± 2°C.

Tobacco plants at the preflower stage were used in the experiments. *H. virescens* eggs were obtained from the University of Arkansas-Fayetteville Insect Rearing Facility.

Effect of Variable PAL Expression on Accumulation of Foliar Phenolics. Phenolic extraction and quantification followed procedures described by Bi et al. (1997). Leaf tissue was excised from plants, immediately frozen in liquid nitrogen, and stored at −80°C for up to one week until processed. Five hundred milligrams of terminal foliage from each line was extracted in 5 ml of 50% methanol at 60°C for 24 hr. The extract was filtered through a 0.45-μm pore Whatman polypropylene filter. The filtrate from each sample was immediately injected for reverse-phase high-performance liquid chromatography (RP-HPLC) by using a Nova-Pak C_{18}, 3.8- × 150-mm stainless steel column (Waters Corporation, Milford, Massachusetts). Details of the chromatographic separation are described by Bi et al. (1997). A Waters 996 photodiode array detector monitored absorbance at 280 nm. The sample peak for chlorogenic acid was identified by comparison of retention time and UV spectrum with an authentic standard (Sigma Chemical Co., St. Louis, Missouri). Five replicate samples for each tobacco line were assayed.

Effect of Phenolics on Growth and Survivorship of Neonates. A terminal leaf of each tobacco line was placed into a 500-ml clear plastic container (Fabri-Kal Co., Kalamazoo, Michigan) lined with moistened filter paper. The filter paper was kept moist to inhibit desiccation of the terminal leaf. Initial assessment indicated that excised leaves had less than 4% water loss during the course of the experiment.

Twenty neonates of *H. virescens* were placed on each terminal and reared for five days at 27°C, 16L:8D. Fresh foliage was provided midway during the experiment. Each treatment was replicated five times. Larval survivorship was recorded daily and larval weight measured on the fifth day.

Effect of Phenolics on Larval Susceptibility to HzSNPV. Three representative tobacco lines, suppressed (6-16 274 T4-1), wild type (C17-2), and overexpressed (10-6T1 OX-10) in PAL, were selected. Neonate *H. virescens* were reared to second instars on a wheat germ-based artificial diet (Chippendale, 1970). These larvae fed for two days on a freshly excised fully expanded terminal leaf of the test tobacco line placed in 500-ml clear plastic containers (Solo Cup Company, Urbana, Illinois) (25–30 larvae/container) lined with moistened filter paper.

Disks (4 mm diam.) were cut from young leaves of tobacco lines with a cork borer, and a dose of 0.1 μl HzSNPV [10 occlusion bodies (OBs) per larva; (Elcar, Sandoz Crop Protection, Des Plaines, Illinois] suspended in 0.1% Triton X-100 was applied to the upper surface of each disk. The bioassay arena was made by embedding plastic grids in a Petri dish (100 mm diam.) with a layer of 4% agar gel–water to create 25 individual cells. Each treatment was replicated

four times over the course of several days. One larva was confined to a cell and fed on the leaf disk for 24 hr. After 24 hr, larvae that consumed the entire disk were transferred to 30-ml plastic cups (Solo Cup Company, Urbana, Illinois) with a layer of 5% agar to retain moisture. Larvae were then reared on leaves of the same line for 10 days. Foliage was changed on alternate days. Environmental conditions for the bioassay were identical to the previous experiment. Larval survival was recorded daily. Dead larvae were examined under a phase contrast microscope to confirm HzSNPV-related infection.

Effect of Chlorogenic Acid in Artificial Diet on Larval Weight. Neonate *H. virescens* were reared to second instar on artificial diet with three levels (3000, 400, and 160 μg/g diet; wet weight) of chlorogenic acid in 500-ml clear plastic containers (25–30 larvae/container). Chlorogenic acid was incorporated into diet when the diet temperature was cooled to 50°C. Individual second instars were then transferred to 30-ml plastic cups containing artificial diet with respective levels of chlorogenic acid. Environmental conditions for the bioassay was identical to first bioassay experiment.

Effect of Chlorogenic Acid in Artificial Diet on Larval Susceptibility to HzSNPV. Neonate *H. virescens* were reared to second instar on artificial diet with three levels (3000, 400, and 160 μg/g diet) of chlorogenic acid. Larvae were maintained in 500-ml clear plastic containers (25–30 larvae/container), dosed with HzSNPV (10 OBs per larva), and transferred to 30-ml plastic cups containing artificial diet with the various levels of chlorogenic acid. The bioassay procedures were exactly the same as in the study of the effect of phenolics on larval susceptibility to HzSNPV, except instead of leaf disks, similar size artificial diet disks were used. Larval survival was recorded daily. Environmental conditions for the bioassay were identical to first bioassay experiment. Weights of the surviving larvae were not recorded because many of the HzSNPV-treated larvae died.

Data Analyses. Mortality data were subjected to arc-sin transformation and were analyzed for significance by ANOVA procedures of ProStat (1996). Survival times of HzSNPV-treated larvae fed on the three tobacco lines were calculated, and the means were compared with t test procedures (ProStat, 1996). Larval survivorship, weight, and mortality data for each replicate were plotted against the levels of chlorogenic acid in tobacco lines or in diet, and logarithmic regression relationships were analyzed to determine if larval survivorship, weight, and mortality were dependent upon chlorogenic acid level.

RESULTS

Effect of Phenolics on Larval Survivorship on Tobacco. Chlorogenic acid varied from 163.1 to 3009.6 μg/g fresh leaf in different tobacco lines (Table

TABLE 1. SURVIVORSHIP AND WEIGHT OF *H. virescens* NEONATES ON TRANSGENIC TOBACCO LINES[a]

Tobacco lines[b]	Chlorogenic acid level (μg/g fresh leaf)	Larval weight (mg)	Larval survivorship (%)
C17-1	961.6 (44.5)[c]	2.8 (0.6)[c]	48
C17-2	400.7 (29.7)	3.0 (0.7)	40
C17-3	672.3 (116.9)	3.3 (0.9)	38
6-16 274 T4-1	163.1 (20.9)	2.4 (0.70)	34
6-16 274 T4-2	542.4 (87.0)	1.3 (0.5)	32
160 P3-1	411.9 (3.2)	4.4 (0.7)	20
160 P3-2	201.4 (17.2)	3.1 (0.9)	30
160 P3-3	660.5 (90.7)	1.7 (0.5)	36
104 T5	1184.1 (144.9)	4.9 (0.8)	70
10-6T1 OX-10	3009.6 (66.5)	3.8 (0.7)	56
10-6T1 OX-11	1509.8 (102.2)	3.3 (0.9)	42
10-6T1 OX-18	1998.2 (118.9)	4.2 (0.7)	64

[a]Neonates were reared on tobacco leaves for five days.
[b]OX lines are PAL overexpressed, T4 and P3 lines are PAL-suppressed, and C17 lines are control, wild type. T5 line is a fifth generation line that recovered from PAL suppression.
[c]Numbers in parnetheses are standard errors of means.

1). Neonate survival fed transgenic tobacco lines varied from 20% on the PAL suppressed P3-1 line containing only 411.9 μg/g chlorogenic acid to 70% on the overexpressed 104 T5 line containing more than 1184.1 μg/g chlorogenic acid. Neonate survival was positively ($P < 0.05$) correlated with the level of chlorogenic acid [($r^2 = 0.53$, $N = 12$, $y = 36.20 + 12.02 \ln(x)$] (Table 1).

Effect of Phenolics on Larval Growth on Tobacco. Larval weights on the fifth day ranged from 1.3 to 4.9 mg and were not significantly correlated with chlorogenic acid levels ($P > 0.05$). However, larval weights were generally lowest on the PAL-suppressed T4-1, T4-2, and P3-3 lines (Table 1).

Effect of Phenolics on Larval Susceptibility to HzSNPV on Tobacco. Viral mortality was not observed in larvae fed untreated leaves. As chlorogenic acid levels increased in three transgenic lines, there was a positive correlation with larval mortality on HzSNPV-treated leaves [$P < 0.05$; $r^2 = 0.54$, $N = 11$, $y = 17.79 + 3.70 \ln(x)$]. Mean survival time of HzSNPV-treated larvae varied from 5.1 to 5.9 days among the lines. Larval survival time was positively correlated with the level of chlorogenic acid in the tobacco lines [$P < 0.05$; $r^2 = 0.98$, $N = 3$, $y = 3.75 + 0.27 \ln(x)$]. Larvae fed tobacco lines OX-10 and C17-2 having higher amounts of chlorogenic acid also had significantly ($P < 0.05$) higher survival time than larvae on T4-1 plants (Table 2).

Effect of Chlorogenic Acid in Artificial Diet on Larval Growth. After 11 days, larval weights on the different diet treatments ranged from 7.1 to 162.0 mg and varied significantly in the order of 160 > 400 > 3000 μg chlorogenic

TABLE 2. SURVIVAL TIME AND MORTALITY OF SECOND-INSTAR *H. virescens* ON TRANSGENIC TOBACCO LINES

Tobacco lines	Chlorogenic acid level (μg/g fresh leaf)[a]	Larval survival time (days)[b]		Larval mortality (%)[b]	
		Untreated larvae	HzSNPV-treated larvae	Untreated larvae	HzSNPV-treated larvae[c]
OX-10	3009.6	>10	5.9 (0.3)[b]a[e]	0	46.8 (1.8)[d]a[e]
C17-2	400.7	>10	5.4 (0.3) a	0	43.0 (3.4) a
T4-1	163.1	>10	5.1 (0.2) b	0	30.5 (2.0) b

[a]Data from Table 1.
[b]Neonates reared on artificial diet to second instar, fed for 24 hr on either untreated or HzSNPV-treated (10 OBs/larva) leaves, and reared for 10 days on respective tobacco leaves.
[c]Arc sin transformed means.
[d]Figures in parentheses are standard errors of means.
[e]Means in a column followed by same letter are not significantly different ($P > 0.05$).

acid/g ($P < 0.05$). Larval weight decreased with increasing levels of dietary chlorogenic acid [$P < 0.05$; $r^2 = 0.89$, $N = 12$, $y = 401.19 - 49.96 \ln(x)$] (Table 3).

Effect of Chlorogenic Acid in Artificial Diet on Larval Susceptibility to HzSNPV. Larval mortality was not observed on untreated chlorogenic acid diet. Mortality of larvae fed on virus-treated chlorogenic acid incorporated diet and reared on similar non-treated diet ranged from 37.4 to 56.0%. Larval mortal-

TABLE 3. EFFECT OF DIETARY CHLOROGENIC ACID ON LARVAL WEIGHT, SURVIVAL TIME, AND MORTALITY OF SECOND-INSTAR *H. virescens*

Chlorogenic acid level (μg/g diet)	Larval weight (mg)		Larval survival time (days)		Larval mortality (%)	
	Untreated larvae[a]	HzSNPV-treated larvae	Untreated larvae	HzSNPV-treated larvae[b]	Untreated larvae	HzSNPV-treated larvae[b]
3009.6	7.1 (0.9)[c]c[d]	NA[e]	>10	5.8 (0.2)[c]a[d]	0	37.4 (2.9)[c]b[d]
400.7	81.1 (6.4) b	NA	>10	5.3 (0.1) b	0	49.1 (3.9) a
163.1	162.0 (10.7) c	NA	>10	5.7 (0.1) ab	0	56.0 (4.8) a

[a]Neonate reared on artificial diet for 11 days.
[b]Means were transformed by arc sin prior to analysis.
[c]Figures in parentheses are standard errors of means.
[d]Means in a column followed by same letter are not significantly different ($P > 0.05$).
[e]Since in all treatments many of the HzSNPV-treated larvae died, weights of the remaining larvae were not taken.

ity was significantly negatively correlated with the levels of chlorogenic acid in diets [$P < 0.05$; $r^2 = 0.56$, $N = 12$, $y = 87.28 - 6.26 \ln(x)$] (Table 3). Mean survival time of HzSNPV-treated larvae varied from 5.3 to 5.8 days among treatments. Larval survival time was not correlated with the level of chlorogenic acid ($P > 0.05$). Larvae fed the highest chlorogenic acid concentration had significantly higher survival time than larvae on the medium concentration ($P < 0.05$) (Table 3).

DISCUSSION

Our data do not support the role of phenolics such as chlorogenic acid in mediating foliar resistance to *H. virescens* or inhibiting larval susceptibility to HzSNPV. Previously, chlorogenic acid was shown to have either negative effects (Elliger et al., 1981; Isman and Duffey, 1982; Felton et al., 1989; Stamp et al., 1994; Yang et al., 1996) or no effect (Lindroth and Peterson, 1988) on larval growth of lepidopterans. Our study showed that chlorogenic acid in transgenic tobacco lines was positively correlated with larval survivorship and susceptibility of *H. virescens* to HzSNPV. We recently reported that larval survival of third-instar *H. virescens* was unaffected by the level of PAL expression in these tobacco lines (Bi et al., 1997). These studies indicate a difference in effects between first and third instars.

Previous studies on the role of phenolics in mediating susceptibility of insects to virus have employed artificial diets supplemented with phenolics (Felton et al., 1987; Keating et al., 1989) or relied upon correlation to assess associations between foliar chemistry and susceptibility (Keating et al., 1988; 1990). The obvious limitation with artificial diets is that it is impossible to realistically simulate the natural chemical milieu of plant tissue. This is especially problematic for phenolics where they may act as antioxidants (Ahmad, 1992, 1995) or in other instances as prooxidants (Ahmad, 1992; Summers and Felton, 1994), depending upon the physico-chemical context of the host plant tissue and the larval digestive system (Appel, 1993, 1994; Johnson and Felton, 1996). Plant tissues contain factors that may accentuate (e.g., phenol oxidases, laccases, peroxidases) (Felton et al., 1989; Appel, 1993) or attenuate (e.g., ascorbic acid, glutathione) (Summers and Felton, 1994) phenolic toxicity. Phenolic oxidation and redox cycling produces reactive oxygen species that may inhibit viral infectivity by one of two mechanisms: (1) by directly inactivating viruses and/or (2) by causing damage to the midgut epithelium and increasing sloughing of midgut cells that subsequently reduces successful viral replication in these target tissues (Hoover et al., 1998c). Consequently, the ability of phenolics to mediate resistance depends upon the particular insect species and the inter- and intraspecific variation in the chemical context of the host plant (Duffey et al., 1995). In this

example (tobacco, *H. virescens*, and HzSNPV), phenolics may act as antioxidants and thus protect the virus from inactivation by reactive oxygen species. Verification of this hypothesis requires further experimental evidence.

While phenolics in tobacco are not directly harmful to lepidopteran larvae (see concepts outlined by Duffey et al., 1995), there are other plant species where the enzymatic activity of polyphenol oxidase or peroxidase are higher and/or the levels of antioxidants lower. In these systems (e.g., cotton), phenolics may be more biologically active (Hoover et al., 1998b). Perhaps in tobacco phenolics do not act as prooxidants because of high antioxidant levels.

We caution against dismissing phenolics as causal factors in mediating larval resistance or susceptibility to viral disease. The reasons for the disparity with earlier studies that implicated phenolics in herbivore resistance (e.g., Felton et al., 1989) and viral susceptibility (Felton et al., 1987) are not immediately clear. Several factors may complicate the ability to clarify the roles of phenolics. For instance, although the tobacco lines are nearly isogenic, the phenotypic expression of other plant defenses may be altered. For example, increased expression of phenolics may result in the down-regulation of other defense pathways or act as metabolic sinks to limit the synthesis of other metabolites. Increased accumulation of salicylic acid in the overexpressed lines may inhibit the biosynthesis of jasmonic acid (Felton et al., 1999), an important signal for the expression of systemic wound response proteins (protease inhibitors, polyphenol oxidases, lipoxygenases, etc.) (Peña-Cortez et al., 1993; Conconi et al., 1996). Jasmonate also activates nicotine biosynthesis in tobacco (Baldwin, 1996).

We found that HzSNPV-infected *H. virescens* larval survival time and susceptibility to baculovirus showed a positive association on tobacco lines. In contrast, Alvarez and Garcia (1992) reported that LT_{50} value of virus-infected *S. littoralis* (Boisduval) larvae increased as the susceptibility of the larvae to virus decreased on different host plants. However, previous results on effects of nutrition on the incubation period of virus infected larvae were inconclusive. High content of dietary protein enhanced *H. virescens* larval susceptibility, but attenuated *Trichoplusi ni* (L.) larval susceptibility to baculoviruses (Hoover et al., 1997). Thus, these studies indicate an idiosyncratic relationship between larval growth and rate of viral mortality.

Our study highlights the difficulty in establishing the causal roles of natural products in mediating plant-herbivore–natural enemy interactions. Although there is solid evidence supporting a causal role of phenolics in mediating pathogen resistance in plants (Maher et al., 1994), clarifying the role of phenolics in plant–herbivore interaction requires further investigation.

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NEMATICIDAL ACTIVITY OF QUINOLIZIDINE ALKALOIDS AND THE FUNCTIONAL GROUP PAIRS IN THEIR MOLECULAR STRUCTURE

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Abstract—Nematicidal activity of aloperine, cytisine, *N*-methylcytisine, and matrine was bioassayed by the agar medium method and the cotton ball method. The corresponding results from the two methods showed that the nematicidal activity of aloperine was the strongest. Its activity, expressed as log $(1/ID_{50})$ was also the strongest (8.67) in comparison with the previously reported nematicidal activity of seven other quinolizidine alkaloids bioassayed by the same method. We hypothesize that the nematicidal activity of quinolizidine alkaloids is determined primarily by the types of functional group pairs and types of functional group in their molecular structures. Based on this hypothesis, alkaloids with strong nematicidal activity can be predicted on the basis of their structure.

Key Words—*Bursaphelenchus xylophilus*, quinolizidine alkaloid, nematicidal activity, functional group pair, pine wilt disease, molecular structure, bioassay, *Sophora alopecuroides* L.

INTRODUCTION

Since pine wilt disease was discovered in Nanjing in 1982 (Cheng et al., 1983), it has spread rapidly in China and become a serious problem for pine forests in China. There has been no effective means to control the disease. Although there are several registered nemacides in Japan, they are used only to protect valuable trees in parks because of their high cost (Jiang, 1994).

Quinolizidine alkaloids are found mainly in Leguminosae plants and represent about 2% of the known alkaloids from these plants (Wink, 1987). This group of alkaloids can be divided into about 10 types according to their structures (Kinghorn and Balandrin, 1984). We have separated and identified more than 10

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quinolizidine alkaloids from *Sophora alopecuroides* (Zhao, 1980; Wang et al., 1986, 1991), the structures of which belong to matrine, cytisine, and aloperine types. Two bioassay methods were used to investigate the relationship between molecular structure and nematicidal activity and compare the nematicidal activities among aloperine, cytisine, *N*-methylcytisine, and matrine, and to compare the advantages and disadvantages of the two bioassay methods. Currently there is no universal theory describing the relationship between molecular structure and the bioactivity of nitrogenous organic compounds. However, it is well known that the nitrogenous functional groups in those compounds are involved in their biological activity. The relationship between molecular structures and bioactivities of eight quinolizidine alkaloids with known nematicidal activity has been explained satisfactorily based upon the concept of functional group pairs. The results are reported in this paper.

METHODS AND MATERIALS

The Pine Wood Nematode. The pine wood nematode used in this experiment was obtained from a colony cultured in our laboratory for three years. It was originally collected from dying pine trees in Nanjing, P. R. China and identified as *Bursaphelenchus xylophilus* (Steiner et Buhrer) Nickle (Zhao, 1996).

Extraction of Crude Alkaloid Fraction from S. alopecuroides. Dried aerial parts of *S. alopecuroides* (5 kg) collected during the flowering stage in Yanchi County, Ningxia, were ground and extracted with three 10-liter aliquots of 75% EtOH. The extracts were pooled, and pastelike extracts were obtained after the solvent was recovered. The pastelike extracts were dissolved in 1% HCl to which 400 ml water was added. The solution was extracted with an equal volume of chloroform and the chloroform solution was discarded. NaOH (5%) was added to the water layer to give pH 10, and the aqueous layer was extracted with an equal volume of chloroform until no alkaloids were found by thin-layer chromatography. After pooling and evaporating the chloroform extracts, 158 g total alkaloids were obtained as a brown paste.

Isolation and Identification of Aloperine, Cytisine, N*-Methylcytisine, and Matrine.* The alkaloid fraction was dissolved in 200 ml of methylbenzene and partitioned with 2 liters of water twice, and 96.5 g of alkaloids were obtained by evaporating the methylbenzene extracts. The water layers were combined and evaporated under reduced pressure to 400 ml, and extracted three times with an equal volume of chloroform, yielding 45.4 g of alkaloids after combining and evaporating the chloroform extracts.

One gram of the alkaloids from the methylbenzene fraction was applied to an alumina column (200 g, activity II), and fractions were eluted with chloroform and chloroform–methanol (100 : 1, 100 : 5, 10 : 1, 2 : 1, and 1 : 1). Five monomers were obtained, the second being monomer B (0.125 g).

One gram of the alkaloids from the water portion was applied to another alumina column (200 g, activity II), and the column was eluted with chloroform–methanol (2 : 1, 1 : 1, 1 : 2, and 1 : 1) and methanol. Four monomers were obtained, of which the first to third were F, G, and H (0.249, 0.015, and 0.0011 g, respectively).

To identify the above alkaloids, we used the following instruments and conditions. Melting point was measured with a thermometer (scales were not corrected). Optical rotation of the alkaloids was measured by WZZ1 OR meter (Shanghai Shenguang Optical Instrument Factory). IR spectra were measured by IR-440 (Shimadzu Co.). MS data were given by GC-MS (Hewlett Packard 5989A), column: DB-O, 30 m × 25 mm ID × 0.15 μm film thickness, temperature program: 100°C for 1 min to 250°C at 15°C/min, injector 250°C, 70 eV.

The physiochemical data measured for the F, G, H, and B monomers (Table 1) agreed with those from references shown in Table 1. Moreover, IR spectra of the monomer H and B were compared with standard sample *N*-methylcytisine and matrine (a gift from Dr. Zhaojing Yan, Ertoke Pharmaceutical Plant, Inner Mongolia, P. R. China) and the spectra were identical to the known compounds. Therefore, the monomers F, G, H, and B were identified as aloperine, cytisine, *N*-methylcytisine, and matrine, respectively.

The Cotton Ball Method. A cotton ball (5 mm diam.) containing a known dose of the test alkaloid was prepared by injecting 100 μl of a methanol solution of the compound into the ball, which was dried under reduced pressure.

A fungal mat of *Botrytis cinerea* was made from a culture of the fungi grown at 22°C for four days on PDA agar medium (3 ml) in a Petri dish (4 cm ID) and the cotton ball was placed in the center of the mat. Then 100 μl of an aqueous suspension of cultured nematodes (15,000 nematodes/ml) were pipetted into the cotton ball and the test culture was incubated at 26°C. After five days the living nematodes were separated from the culture through the tissue paper in the Baermann funnel and killed by heating in boiling water. The nematacidal effect was calculated from the number of nematodes in the test culture and the number in the control culture as follows:

$$\text{nematicidal effect} = [1 - (\text{number in test culture}/\text{number in control culture})] \times 100$$

The ID_{50} (moles per cotton ball) indicates the dose of an alkaloid at which the propagation seen in the control was inhibited by 50% and was calculated by probit transformation of the nematicidal effect (Matsuda et al., 1991). Expression of nematicidal activity as an index, calculated as log $(1/ID_{50})$, allowed comparison with previously published results. Details of the cotton ball method can be found in Mastuda et al. (1989, 1991).

TABLE 1. PHYSIOCHEMICAL AND GC-MS DATA USED TO IDENTIFY ALKALOIDS FROM *S. alopecuroides*

Alkaloid	MP (°C)	$[\alpha]_D$	MS *m/z* (%)	IR (cm^{-1})	Reference
F, aloperine	70–71	+36.5°(26°C = 1.27, EtOH)	323(M^+, 40), 203(3), 189(20), 174(15), 148(19), 134(32), 98(100), 97(28), 96(46), 84(16)	2920 1160	Dokachev et al. (1975)
G, cytisine	152–154	−109.2°(25°C, C = 1.06, EtOH)	190(M^+, 82), 160(28), 147(96), 146(100), 134(27)	1642 1543 1520	Wink (1993) Zhong (1983)
H, *N*-methyl-cytisine	135–136	−226°(22°C, C = 1.26, H_2O)	204(M^+, 24), 160(150), 146(12), 146(100)	2760 1645 1665 1543	Zhao (1980) Silva (1968) Okuda et al. (1965) Bohlmann et al. (1958)
B, matrine	75–76	+38.2°(26°C, C = 0.51, H_2O)	248(M^+, 100), 247(89), 205(64), 150(54), 137(32), 96(60)	2930 2850 2800 2755 1625	Okuda et al. (1965) Morinaga et al. (1978)

Agar Medium Method. A fungal mat of *Botrytis cinerea* was formed at 22°C for five days on 8 ml of PDA agar medium in a Petri dish (5 cm ID), into which was pipetted 200 μl of a known dose of the test alkaloid. Then 100 μl of an aqueous suspension of cultured nematodes (10,000 nematodes/ml) were added to the mat and incubated at 25–26°C for five days. Nematodes were separated from the culture by the funnel method and counted under a microscope.

HPLC analysis showed that there was no significant difference in the structure and the dose of the test alkaloids in the PDA agar medium before and after steam sterilization (121°C). The nematicidal effect was calculated by the same formula given earlier. Additional details of the bioassay can be found in Zhao (1996).

RESULTS

Table 2 shows the nematicidal activities of the four alkaloids assayed by the cotton ball method, and Table 3 shows data from the agar medium method after five days incubation. Aloperine was the most effective alkaloid.

TABLE 2. NEMATICIDAL EFFECT AND NEMATICIDAL ACTIVITY OF FOUR ALKALOIDS AGAINST PINE NEMATODES BIOASSAYED BY COTTON BALL METHOD

Alkaloid	Nematicidal effect[a] (μg/cotton ball)							Nematicidal activity $\log[1/ID_{50}]$	Order of nematicidal activity
	0.1	0.3	1.0	3.0	10	30	100		
Aloperine		100.0	100.0	98.0	80.1	28.2	3.5	8.67	1
Cytisine		100.0	96.0	65.3	47.6	15.7	2.0	8.18	2
N-Methylcytisine		100.0	91.5	56.5	16.4	1.0	0	7.87	3
Matrine	89.7	16.2	7.9	1.1	0	0		6.28	4

[a]See Matsuda et al. (1991).

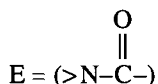
TABLE 3. NEMATICIDAL ACTIVITY OF FOUR ALKALOIDS ASSAYED WITH AGAR MEDIUM METHOD ISOLATED FROM *S. alopecuroides*

Alkaloid	MW	Molar concentration	(% Mortality ± SD)
Aloperine	230	4×10^{-4}	100.00 ± 0.00
Cytisine	190	4×10^{-4}	48.06 ± 0.83
N Methylcytisine	204	4×10^{-4}	34.35 ± 0.57
Matrine	247	4×10^{-4}	23.06 ± 1.21

DISCUSSION

The results of the two bioassay methods showed that the order of nematicidal potency of the four alkaloids was the same in both assays, with aloperine showing the strongest effects and the matrine the weakest effects. Table 4 compares the relative nematicidal activities of aloperine and seven additional quinolizidine alkaloids against the pine wood nematodes in comparison to activities related in the literature.

Matsuda et al. (1989, 1991) reported the relationship between molecular structures of quinolizidine alkaloids and their activity against pine wood nematodes and suggested that the cause of nematicidal potency of the cytisine-type structure was the α-pyridone ring in the molecules. However, the nematicidal activities of sophoramine and anagyrine (Figure 1), which have an α-pyridone ring in their molecule, were poor (Table 4). Apparently, the α-pyridone ring is not the main cause of the strong nematicidal activities of quinolizidine alkaloids (Mastuda et al., 1991). Morita and Oka (1979) investigated with some success the relationship between physiological activities of synthetic organic nitrogenous compounds in humans and their molecular structures by using the concept of functional group pair (FGP). FGP refers to two functional groups connected by two or three carbon atoms. For examples, there are five nitrogenous functional groups (FGs), designated A–E, as follows: A = (–NH_2), B = (–NH–), C = (>N–), D = (>N^+ <), and



and two FGs, designated F and G, containing oxygen: F = (–OH), G = (–O–). Therefore, if a number is used to stand for the number of carbon atoms between the two FGs, abbreviations such as B2C and C3E can be used to stand for FGP. For example, C3A stands for >N–C–C–C–NH_2, where C indicates the first nitrogen atom that is separated by three carbon atoms from the second nitrogen containing group. Similarly, E3B stands for

TABLE 4. STRUCTURE TYPE, FGP TYPE, AND NEMATICIDAL ACTIVITY OF THE QUINOLIZIDINE ALKALOIDS

Alkaloid	Type of structure[a]	FGP	Double bonds (*N*)	Nematicidal activity[b] log (1/ID 50 index)		Order of nematicidal activity
				Data from literature	Data from this paper	
Aloperine	Aloperine	B3C	1		8.67	1
Cytisine	Cytisine	B3E	2	8.23 (Matsuda et al., 1989)	8.18	2
Sparteine	Sparteine	C3C	0	7.96 (Matsuda et al., 1991)		3
N-Methylcytisine	Cytisine	C3E	2	7.91 (Matsuda et al., 1989)	7.87	4
Sophocarpine	Matrine	C3E	1	7.78 (Matsuda et al., 1991)		5
Anagyrine	Anagyrine	C3E	2	7.54 (Matsuda et al., 1989)		6
Sophoramine	Matrine	C3E	2	6.68 (Matsuda et al., 1991)		7
Matrine	Matrine	C3E	0	6.39 (Matsuda et al., 1991)	6.28	8

[a]See Kinghorn et al. (1984).
[b]The cotton ball bioassay method was used in all cases.

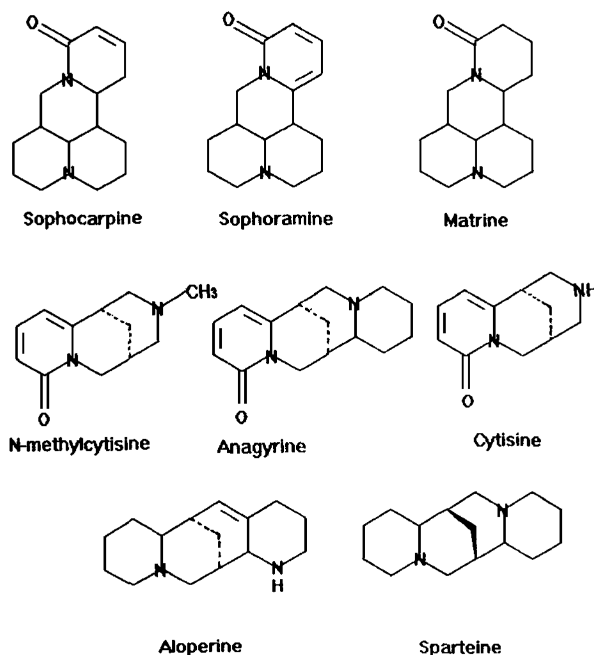
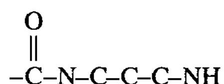


FIG. 1. Quinolizidine alkaloids.



An hypothesis relating structure of quinolizidine alkaloids with their nematicidal activity against pine wood nematodes is proposed based on the bioassay results shown in Table 4, and is described below.

1. Nematicidal activity of a quinolizidine alkaloid against pine wood nematodes depends mainly on its FGP type. For example, from Table 4, the activity of aloperine with B3C type is stronger than that of cytisine with B3E; and the activity of sparteine with C3C type is greater than that of any of the other remaining five alkaloids. However, the structure type seems not to be the decisive factor for determining nematicidal activity, since an alkaloid of sparteine type appears between those of cytisine type and an alkaloid of anagyrine type exists between those of matrine type, respectively, in Table 4, in which alkaloids are arranged in order of their nematicidal activity. The difference in nematicidal effect between sophoramine and matrine with the same matrine type of structure is 1.39. Moreover, although the nematicidal activity of aloperine is the strongest, that of

Δ-dehydroaloperine with the same aloperine type of structure is very weak, and similar to matrine (unpublished data).

2. Activity of an individual type of FGP is dependent on the sum of activities of the two FGs in it; an individual FG makes certain contributions to the activity of the molecule, and the activity of the FGP depends on the sum of the activities of the two FGs in the FGP.

Based on data from Table 4, a group of equations

$$(1)\ B + C = 8.67, (2)\ B + E = 8.23,$$
$$(3)\ C + E = (7.91 + 7.78 + 7.54 + 6.68 + 6.39) \times 1/5$$

is set up and solved to get B = 4.82, C = 3.85, and E = 3.41. Thus, B > C > E. Therefore, B + C > B + E, and C + C > C + E. This order is in agreement with the observed activities in Table 4 and is consistent with the activity relationships among the structures of the eight alkaloids in Table 4.

Because too few alkaloids with B3E or C3C FGP were bioassayed, it is not possible at present to draw a conclusion on relative potency of the alkaloids with these two FGPs.

3. Clearly, the three dimensional structure, position, and number of double bonds of alkaloids affect their nematicidal activity, but generally those factors are secondary. However, for alkaloids with the same FGP, they become the determining factors. For instance, because of the presence or absence, position, and number of double bonds present, matrine, sophocarpine, and sophoramine with the same configuration (see Figure 1) have different nematicidal activity.

The hypothesis remains to be tested further and improved with additional experimental results. We predict that other alkaloids with the functional group pairs B3C, and possibly those with B3B, will possess greater nematicidal activity against pine wood nematodes.

The agar medium bioassay method reflects the conditions occurring during field application of alkaloids to control the pine wood nematode because the nematodes are continuously in contact with the alkaloids. In contrast, in the cotton ball bioassay, once the nematodes leave the cotton ball and enter the hyphae of *B. cinerea*, they are no longer in contact with the alkaloids. An important advantage of the cotton ball method, however, is the low dosage required. It is a useful method for those alkaloids that are available in small quantities either because they are difficult to separate and purify or because they are contained in plants in trace amounts.

The results from the two bioassay methods show that aloperine is the most active alkaloid against pine wood nematodes. Aloperine is also the most potent alkaloid among those tested with known nematicidal activity. Moreover, there are rich resources of wild *S. alopecuroides* in China, and small-scale field tests indicated that aloperine can effectively control pine wilt decease. With further

improvement in the application method, aloperine promises to become an effective natural compound to control pine wilt disease.

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THE CHEMISTRY AND MINERALOGY OF THREE SAVANNA LICK SOILS

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Abstract—Three lick soils were sampled from the Mkomazi Game Reserve, Tanzania, and subjected to geochemical and mineralogical analysis. Compared to 88 samples of topsoil collected for a reconnaissance geochemical soil survey, the lick soils are geochemically distinct, being either enriched in certain constituents (e.g., total and extractable Na) or depleted in others (e.g., the base cations) depending on the lick soil considered. There are notable differences in the geochemistry and mineralogy of the lick soils to the extent that no single characteristic of these soils can explain their exploitation. One lick soil, enriched in kaolinite, could act as a pharmaceutical agent by preventing or treating gastrointestinal upsets. Two lick soils are enriched in Na, which may explain their utilization, although both soils to a lesser or greater extent additionally contain constituents such as $CaCO_3$ and smectite, which can alleviate acidosis (and smectite can act as a pharmaceutical agent, similar to kaolinite). One of these soils, an extremely alkaline, highly calcareous, and saline–sodic soil is particularly distinctive and may provide a range of benefits if consumed in appropriate amounts.

Key Words—Geophagy, salt lick, soil geochemistry, soil mineralogy, Tanzania.

INTRODUCTION

Geophagy, the deliberate ingestion of soil, is very common in the Animal Kingdom (Beyer et al., 1994). Often the soil intake is selective, concerning the exploitation of specific sites and sometimes even particular soil horizons. These sites are variously named mineral licks, natural licks, or salt licks (Kreulen, 1985). Such terminology is unfortunate since the words "mineral" and "salt" suggest that the animals are eating soil to satisfy a mineral nutrient imbalance.

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However, while the literature strongly supports the view that Na is important in the causation of deliberate soil consumption [with, for example, herbivorous mammals possessing a Na-specific perception and hunger mechanism that is activated by Na depletion (Denton, 1969; Dethier, 1977)], the evidence for the other mineral nutrients is less compelling (Kreulen and Jager, 1984). Indeed the possible causes of geophagy are manifold. For example, there is evidence to suggest or show that soil may be deliberately consumed to counteract acidosis in wild ruminants/ungulates, to adsorb ingested plant toxins in primates, to dispel hunger during starvation (which certainly has been undertaken by humans), and to break down food in birds, reptiles, and ruminants by abrasion (Ayeni, 1971; Bolton et al., 1998; Halsted, 1968; Kreulen, 1985, Miller et al., 1977). However, despite the widespread occurrence of geophagy, the literature relating to this subject remains limited, largely speculative, and fragmentary, which contributes to a misunderstanding of the practice.

At the invitation of the Tanzanian Government, the Royal Geographical Society (London) brought together a small interdisciplinary group of specialists to undertake a detailed ecological study of the Mkomazi Game Reserve (MGR) (Coe et al., 1999). As part of the so-called Mkomazi Ecological Research Program (MERP), I undertook a soil geochemical reconnaissance survey of the MGR in September, 1995 (Abrahams and Bowell, 2000). While undertaking this survey, I had the opportunity to examine and sample the three known lick sites that are frequented by animals within the reserve. This paper reports the geochemistry and mineralogy of these soils and compares some of the characteristics with those determined from the soils collected for the reconnaissance survey.

METHODS AND MATERIALS

Location. The MGR was established in 1951 and now covers an area of about 3600 km^2. Generally, the reserve can be considered as a semiarid environment with precipitation ranging from 300 to 900 mm/yr (although the hills, which account for only about 4% of the land area, receive up to 1500 mm/yr). The annual rainfall is split fairly evenly between the "long rains" (March to mid-May) and the "short rains" (late October to December). Mkomazi lies on the Tanzania–Kenya border (Figure 1), with the northern boundary adjacent to the Tsavo (West) National Park in Kenya. The region is thus a joint reservoir of wildlife, which is accommodated in a huge area of (commonly) *Acacia–Commiphora* bushland. Due to Mkomazi's higher rainfall, much of the western and central areas of Mkomazi act as a wet season refuge for wildlife from Tsavo.

Prior to the MERP, ecological and other research in the MGR had been limited. However, the 3-year MERP has produced enough data to confirm the

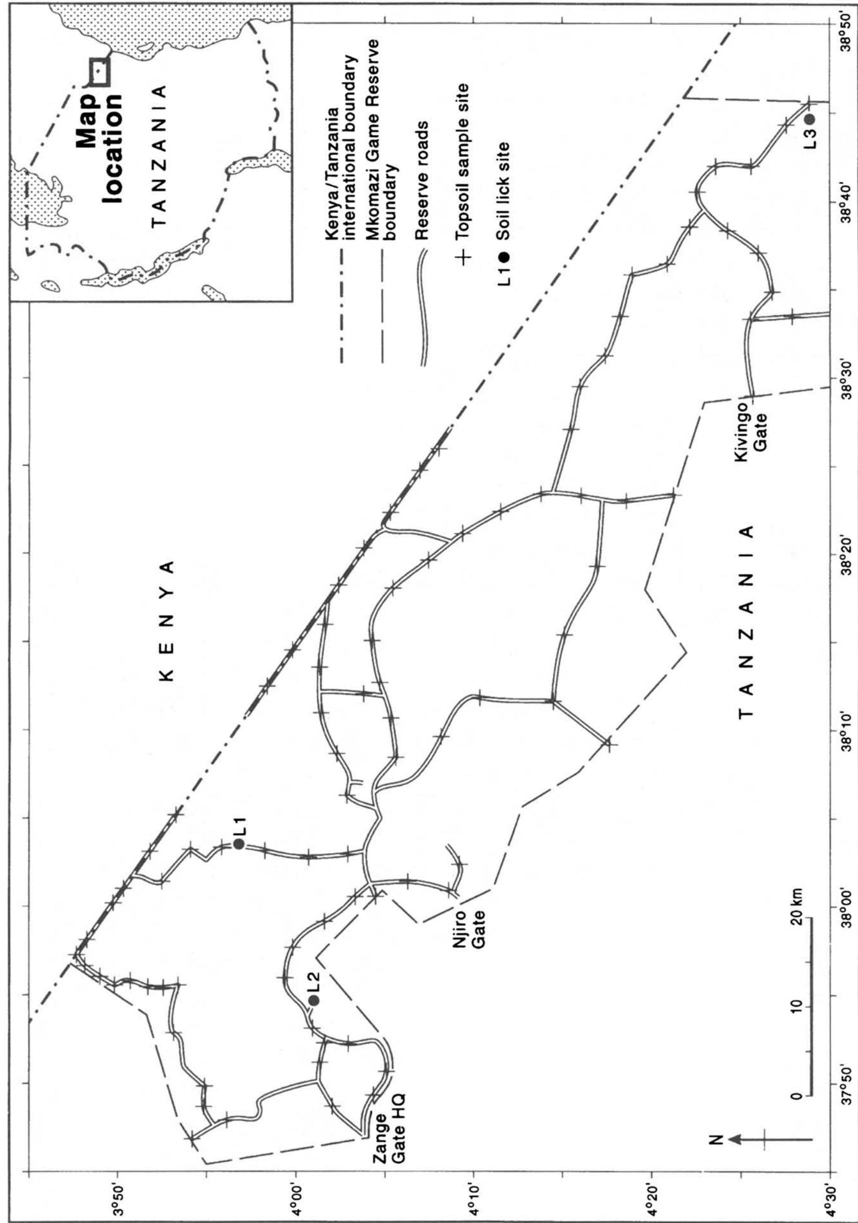

FIG. 1. The Mkomazi Game Reserve, Tanzania.

existence of a diverse and rich flora and fauna. Mkomazi is now recognized as one of the richest savanna habitats in Africa, and probably the world, even though former hunting, poaching and pastoralism have taken their toll in this area on both habitats and wildlife (Coe et al., 1999).

Little work on the geology and soils of the reserve has been published, and the soil maps that do exist are general and have been published at a small scale (Hathout, 1983). Such maps indicate that well drained, loam textured Ultisols predominate, although dark colored, clay-rich soils variously called mbugas, black cotton soils, or Vertisols are common and are associated with seasonally inundated depressions. Locally, areas of saline and alkaline soil are associated with edges of the Umba river (the only permanent water source within the MGR) and seasonal river courses.

Methodology. For the rapid soil geochemical reconnaissance survey, 88 samples of topsoil (0–15 cm depth) were collected throughout the reserve. A Land Rover vehicle equipped with a GPS instrument aided the positional recording of the sample sites (Figure 1). Abrahams and Bowell (2000) provide further details. To assist with this work, the MERP was well supported with rangers who provided practical help and local knowledge. Despite the large size of Mkomazi, the reserve rangers knew of only three lick sites. Each location was visited when the work being undertaken for the rapid soil geochemical survey approached the lick sites (Figure 1). Because the sampling was undertaken in the dry season, no animals were actually observed utilizing the licks since surface water was scarce. Lack of surface waters significantly influences and limits the number of animals within the MGR at this time of the year. However, some recent use of the licks was evident, with elephants clearly exploiting at least two of the sites. Brief details of the study locations follow:

Lick site 1 (L1) is located at 38°3.52′E, 3°56.79′S. This site was immediately adjacent to one of the dirt roads in the game reserve and consisted of a hole that had been excavated by animals. There was nothing obviously distinctive about this site relative to the surrounding soils. The (air-dried) red soil color (Munsell color notation 2.5YR 4/8) is similar to that observed for much of the MGR. A stainless steel trowel was used to collect a sample from the face of the excavated pit.

Lick site 2 (L2) is located at 37°54.66′E, 4°1.00′S. This lick occupies the bank of a seasonal river channel that was dry at the time of sampling. Impressive excavations have been mined along with smaller holes opened by elephant tusks (Figure 2). A sample was collected from one of the excavations. The most obvious distinctive feature of the soil is the color (white 5Y 8/1 to light grey 5Y 7/1), which contrasts with the predominantly red- and brown-colored soils found elsewhere within the reserve.

Lick site 3 (L3) could only be visited on foot and the GPS installed in the vehicle could not be used to record the location accurately. However, the

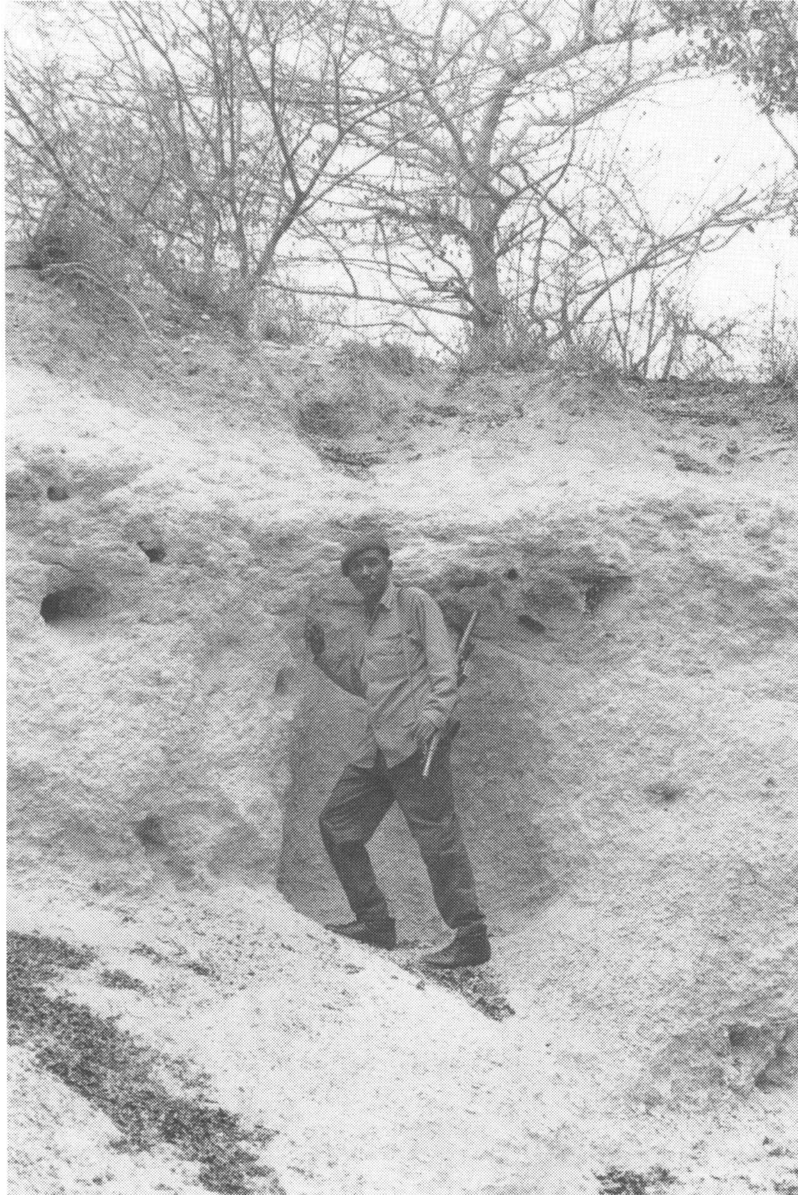

FIG. 2. Soil lick site L2. The reserve ranger is standing in an excavation mined by animals. Other holes made by elephant tusks can be seen. Clearly, it is subsoil material that is being consumed at this locality. The site itself is a bank adjacent to an ephemeral river.

approximate position is indicated in Figure 1; the site is located near the Umba river. The yellowish red soil (5YR 4/6) was distinctly marked by elephant tusks, and unidentifiable fecal droppings further suggested utilization by other animals. A soil sample was collected from the bank of soil marked by the elephant tusks.

Following collection, all the soil samples were stored in Kraft paper bags. On arrival at the laboratory in the UK, the samples were air-dried, disaggregated in a porcelain mortar with a pestle, and passed through a 2-mm nylon mesh sieve. The <2-mm fine earth fraction was then used for the determination of soil pH, organic matter content, salinity, carbonate content, and the total and extractable concentrations of Ca, Co, Cu, Fe (total concentrations only), K, Mg, Mn, Na, Ni, P, Pb, and Zn. Full details of the methodologies employed, and the analytical quality control procedures undertaken to assess the precision and accuracy of the laboratory determinations, are provided by Abrahams and Bowell (2000). Consequently, only brief details of the laboratory methodologies employed are given here:

(1) Soil pH was determined from a 1 : 2.5 soil–water and soil–0.01 M $CaCl_2$ suspension with a pH electrode and meter. Rowell (1994) presents details of measurement.
(2) Organic matter content was determined gravimetrically following the ignition (375°C for 16 hr) of oven-dried (105°C for 16 hr) soil.
(3) Salinity was rapidly appraised in units of dS/m by measuring the electrical conductivity (EC_e) of soil solutions extracted from soil pastes.
(4) Carbonate content was assessed by observing the reaction of soil with a few drops of 10% HCl.
(5) Total element concentrations were determined following a nitric–perchloric acid digestion. This procedure has been called a "powerful attack," effective in solubilizing elements from many soil minerals, although some may be attacked to only a negligible or minor degree; for further details see Thompson and Wood (1982). For the work investigating the 88 topsoil samples, various extractants were used to assess the plant available (or extractable) fraction of elements in the samples. Such extractants are used for the measurement of the water-soluble and the exchangeable (i.e., adsorbed on soil particle surfaces) fractions of elements in soils. The three lick samples were also included in this analysis.

In addition to these methods, the mineralogy of the soil lick samples was determined by X-ray diffraction (XRD). In this analysis, a semiquantitative assessment of the whole-soil mineralogy was undertaken on the fine earth fraction, and a quantitative assessment was performed on the <2-μm (clay) fraction. The results were then combined to give an overall assessment of the soil miner-

alogy. The degree of clay mineral development (e.g., well or poorly crystallized) was evaluated, and the percentage of clay content by weight of the fine earth fraction was determined.

RESULTS

The organic matter content and geochemistry of the lick soils are presented in Table 1. With some exceptions (e.g., percent organic matter content, total and extractable Zn), there is considerable variability among the samples. For example, the pH varies from the strongly acidic reaction of sample L1 (pH 3.8, measured in $CaCl_2$) to the extremely alkaline reaction of L2 (pH 10.7). Sample L3 can be described as moderately alkaline (pH 8.0). For a soil to have a pH above 7, it must either be calcareous (contain calcite, $CaCO_3$), dolomitic (contain dolomite, $CaCO_3 \cdot MgCO_3$), or sodic (contain Na_2CO_3) (Rowell, 1994). An estimate of calcium carbonate content can be determined by observing the reaction of soil with dilute HCl acid (note: this method is less reliable for dolomite). The alkaline soils reacted with this acid to the extent that they can accordingly be described as highly calcareous (sample L2) and slightly calcareous (L3) (Hodgson, 1976). The XRD analysis shows the large amount of calcite in L2, and the presence of dolomite in both L2 and L3 (Table 2). Accordingly, the Ca and Mg concentrations determined from these samples reflect their mineralogy (Table 1). Sodic soils are more alkaline than calcareous soil, and the extreme alkalinity associated with L2 is indicative of sodicity. Again the geochemistry of the sample reflects this, with a total concentration of 12,600 mg Na/kg of which some 94% is extractable (Table 1). While this sample is enriched in Ca and Na, very low total concentrations of Fe, K, P, and, to a lesser extent Cu, are associated with this soil. Similarly, relative to the amounts typically encountered in soils, the lick samples L1 and L3 have some very low or high total concentrations. In this respect, the Pb concentrations are very low in both samples, as is the Ca content of L1. The Na concentration of 5040 mg/kg determined from L3 is high, with much of this element (63%) being in an extractable form.

The mineralogy of the lick samples is displayed in Table 2. Hydrous oxide clays of Fe and Al such as goethite and gibbsite are a constituent of many tropical soils, but none were detected in any of the three lick samples. While this may be a true reflection of the clay mineralogy of the samples (and L2 does contain low amounts of Fe), if the hydrous oxides are poorly crystalline or amorphous, then their detection is difficult by XRD analysis. As with the geochemistry, a feature of the mineralogy is the variability that is evident among the samples. Sample L1 is enriched in clay-sized particles (41.6% by weight), of which poorly crystallized kaolinite dominates. Quartz is the other major mineral found in the fine earth fraction of this soil. Such a mineral composition suggests that this

TABLE 1. ORGANIC MATTER CONTENT AND GEOCHEMISTRY OF LICK SOILS[a]

Sample	pH (water)	pH ($CaCl_2$)	Organic matter (%)	Ca Total	Ca Extr.	Co Total	Co Extr.	Cu Total	Cu Extr.	Total Fe	K Total	K Extr.
L1	4.3	3.8	1.6	280	230	21	3.2	67	5.3	42,000	2,000	225
L2	11.0	10.7	1.0	91,000	360	17	<0.5	8.4	<0.5	8,400	160	7.5
L3	8.2	8.0	1.1	7,450	2,400	17	1.6	34	2.6	29,400	3,300	188

Sample	Mg Total	Mg Extr.	Mn Total	Mn Extr.	Na Total	Na Extr.	Ni Total	Ni Extr.	P Total	P Extr.	Pb Total	Pb Extr.	Zn Total	Zn Extr.
L1	940	88	223	126	120	9	46	5.3	1,250	8	<4.2	1.1	38	<0.3
L2	8,000	13	151	3.2	12,600	11,875	25	0.6	<100	4	17	0.5	29	0.3
L3	5,400	1,500	256	100	5,040	3,188	38	2.1	1,600	<8	4.2	<0.5	46	0.3

[a]Element concentrations in mg/kg. Extr. = extractable.

TABLE 2. MINERALOGY (%) OF LICK SOILS

Sample	Smectite	Illite	Kaolinite	Amphibole	Quartz	K Feldspar	Plagioclase	Calcite	Dolomite	Halite	Pyrite	Total
L1	0.0	0.0	41.0	0.0	52.6	2.4	1.8	0.0	0.0	0.0	2.2	100
L2	11.6	0.0	7.3	0.0	17.8	5.7	26.3	26.1	3.8	1.4	0.0	100
L3	2.3	8.8	12.0	8.7	32.2	10.0	16.9	0.0	1.5	3.7	3.9	100

soil has been subjected to high chemical weathering intensity whereby most primary minerals have been decomposed to secondary kaolinite. Quartz, resistant to weathering, persists in the coarser fractions (silt and sand-sized) of the <2-mm soil. In contrast, L2 and L3 contain a lower proportion of clay-sized minerals (19% and 23.5% by weight, respectively). Sample L2 is dominated by calcite and plagioclase feldspar, and smectite is the main clay mineral. Quartz and feldspars are the major minerals of L3, and illite and smectite account for nearly 50% of the clay mineralogy. The fact that L2 and L3 contain weatherable minerals like feldspars, along with the presence of the clay minerals illite and smectite, indicates that these samples have not been subjected to the same chemical weathering intensity as L1. There may be more than one reason why this has occurred (for example, perhaps the soils of samples L2 and L3 have been protected from such weathering until the removal of the upper part of the soil profile by recent erosion, although this is speculative), but presumably the pH is a factor that is important here, with chemical weathering being accelerated in the strongly acid soil of sample L1 (Brady, 1990).

A feature of the mineralogy of L2 and L3 is the presence of halite in both samples. This mineral is one of a variety of soluble salts that can accumulate in semiarid environments to form saline soils. The salinity of soils can be rapidly appraised by measuring the electrical conductance (EC_e) of soil solutions from saturated soil pastes (Rowell, 1994). Measurement of the electrical conductance of the saturation extract obtained from L2 confirms this sample as moderately saline ($EC_e = 8.5$ dS/m) but L3, with an EC_e of only 0.36 dS/m, is classified as a nonsaline soil. Both L2 and L3 are located on the banks of seasonal or permanent rivers, which accounts for their alkaline and (varying) saline and sodic characteristics.

DISCUSSION

A common practice when undertaking this type of research is to compare the characteristics of the lick samples with nonlick soils found in the same region (e.g., Ayeni, 1972). Differences between the two soil types can then suggest the reasons why animals are deliberately utilizing the lick sites. Following this practice, the three lick soils sampled in this study are compared to the 88 samples of topsoil that were collected for the rapid soil geochemical reconnaissance of the MGR. Abrahams and Bowell (1999) present a detailed appraisal of these topsoil samples. Generally, the topsoils are very low in organic matter (e.g., median = 2.0%, interquartile range = 1.7–2.5%), salt free ($EC_e < 4$ dS/m) and are slightly acidic (median = 5.9 pH units measured in $CaCl_2$). The pH does vary from being strongly acidic (pH 3.5) to alkaline (pH 7.9), with carbonate minerals being a constituent of the latter soils. In contrast, the lick soils contain

even lower amounts of organic matter (which can be explained by these samples being comprised largely of subsoil material) and are more extreme in terms of their reaction. Thus, L1 is, with one exception, more acidic than any other sample collected in the reserve, and L3 and especially L2 are the most strongly alkaline (when measured in a $CaCl_2$ suspension). In other respects the geochemistry of the lick soils are different compared to many of the variables recorded from the topsoil samples. Lick soil L2 is especially distinctive, with the highest total Ca and Na concentrations and lowest amounts of total Fe, K, Mn, and P recorded from anywhere in the MGR. Sample L3 is notably enriched in both total and extractable Na, while L1 has comparatively low concentrations of the base cations Ca, K, Mg, and Na. Overall, of the 78 measurements determined from the three lick soils (Table 1), 50 fall outside the interquartile ranges of the variables determined from the 88 topsoil samples (note: three of these comparisons could not be made because of concentrations that were below the limits of detection).

These differences between the licks and the topsoils sampled throughout the reserve provide some insight as to why the lick soils are being deliberately consumed. Bearing in mind that much previously published work has implicated Na in the cause of geophagy (Kreulen and Jager, 1984), the high concentrations of this element in L2 and L3 are of interest. As indicated previously, a high proportion of this element in these samples is in an extractable form (94% and 63%, respectively), which contrasts with the relatively low amounts (median = 6.6%) of Na in an extractable form in the topsoil samples. These extractable concentrations are determined by conventional reagents used to assess the fraction of an element that is available to plants, and hence they do not necessarily reflect the proportion of an element that is available to the animal once ingested. Nevertheless, the indications are that a significant amount of the Na associated with L2 and L3 would be released into the digestive fluids of animals and so be available for absorption. Similarly, other nutrients such as Ca or Mg could be supplied in significant amounts, even if the animals are not deliberately seeking soils for these elements.

Although licks L2 and L3 contain high concentrations of Na and other mineral nutrients that can alleviate deficiency problems, there may be other beneficial factors that these soils can impart to animals via ingestion. Lactic acid acidosis can afflict wild ruminants/ungulates as well as domestic ruminants and horses at times of sudden dietary changes (Kreulen, 1985). With domestic animals, supplements such as $NaHCO_3$, NaCl, K_2CO_3, $CaCO_3$, and montmorillonite (i.e., a prominent member of the smectite group of clay minerals) provide an antacid function. They can additionally increase energy and nitrogen utilization (Kreulen, 1985). To varying extents, L2 and L3 contain such supplements, with the saline–sodic, calcareous, and smectite-enriched properties of the former being of particular potential value to any animals vulnerable to acidosis.

The chemical and mineralogical composition of L1 is distinctly different from L2 and L3, and this soil would appear to provide no antacid function. Furthermore, for the elements determined in this study, the consumption of this lick soil would seem to offer no significant nutritional benefits since the topsoils within the reserve contain typically the same or even higher total and extractable concentrations. However, sample L1 is enriched in kaolinite, and this clay mineral may be the stimulus for geophagy by wildlife. Kaolin-based pharmaceuticals are commonly used in human populations to treat diarrhea and intestinal upsets (Reynolds, 1993). Previous research has suggested that soils enriched in kaolinite may act as a similar pharmaceutical agent when ingested by nonhuman primates and cattle, preventing or treating gastrointestinal upsets or diarrhea caused by parasites or by poisons and other agents found in forage (Mahaney et al., 1995, 1996a; Knezevich, 1998). It is worth noting that smectite, found in L3 and especially L2, is also used for the treatment of human gastrointestinal disorders (Reynolds, 1993) and may be a stimulus for geophagy by chimpanzees (Mahaney et al., 1996b).

CONCLUSIONS

The explanations for geophagy made above are speculative, and further research is required to either confirm them or find alternative reasons as to why wildlife in the MGR are deliberately ingesting these soils. In this respect this paper is similar to much of the previously published work on this subject, clearly indicating the need for continued research. In particular, there would appear to be a need for more collaborative or integrated studies, linking soil and forage analysis with detailed investigations on the nutrition and health of wild animals. With such animals being increasingly confined to clearly defined and, on occasion, well-fenced reserves, and with the development of conservation and nature-oriented tourism, there will be an increasing need for the sustainable management of game reserves, which will include gaining further understanding on the health of animals. Geophagical studies are clearly important in gaining such an understanding. The practice is widespread within the animal kingdom, and although some problems may occur, such as excessive tooth wear, ingested soils have the potential to provide a range of benefits to wildlife. The analysis of the three lick soils known to occur within Mkomazi illustrates this. These soils differ in a number of respects, not only from the topsoils found within the game reserve, but also among themselves to the extent that no single characteristic considered in this study can explain lick exploitation. One strongly acidic lick soil (L1) has been subject to intensive weathering, with the result that most weatherable minerals have been decomposed to kaolinite. Such a soil could act as a pharmaceutical agent in preventing or treating gastrointestinal upsets. The other two

lick soils, L2 and L3, are notably enriched in Na, a large proportion of which is in an extractable form. These soils also contain constituents such as $CaCO_3$ and smectite, which can provide an antacid function and other benefits. One lick soil (L2) in particular is geochemically and mineralogically distinctive and has the potential of alleviating more than one disorder (e.g., Na deficiency, acidosis).

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SEX PHEROMONE OF *Ascogaster quadridentata*, A PARASITOID OF *Cydia pomonella*

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Abstract—Porapak Q volatile extracts of female *Ascogaster quadridentata*, an egg-larval endoparasitoid of codling moth, *Cydia pomonella*, bioassayed in Y-tube olfactometers attracted male, but not female, *A. quadridentata*. Coupled gas chromatographic–electroantennographic detection (GC-EAD) analysis of bioactive extracts revealed three compounds that elicited responses by male *A. quadridentata* antennae. GC-mass spectra (MS) indicated, and comparative analyses of authentic standards confirmed, that these compounds were (*Z,Z*)-9,12-octadecadienal, (*Z*)-9-hexadecenal, and 3,7,11-trimethyl-6*E*,10-dodecadienal. (*Z,Z*)-9,12-Octadecadienal alone attracted laboratory-reared male *A. quadridentata* in Y-tube olfactometer and field-cage bioassays, and attracted feral *A. quadridentata* in a field experiment. This sex pheromone could be used to help detect populations of *A. quadridentata*, delineate their distributions, and determine potential sources of parasitoids for capture and release in integrated programs for control of *C. pomonella*.

Key Words—Sex pheromone, parasitoid, *Ascogaster quadridentata*, *Cydia pomonella*, (*Z,Z*)-9,12-octadecadienal, (*Z*)-9-hexadecenal, 3,7,11-trimethyl-6*E*,10-dodecadienal, dihydrofarnesal, Braconidae, Tortricidae.

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INTRODUCTION

Ascogaster quadridentata Wesmael (Hymenoptera: Braconidae) is a solitary egg-larval koinobiont endoparasitoid. It parasitizes a large number of microlepidoptera primarily from the family Tortricidae, including the codling moth, *Cydia pomonella* L., a severe pest of apple crops throughout the world (Clausen, 1978).

Searching for mates poses a challenge for solitary parasitoids, such as *A. quadridentata*, especially when found at low densities, as mates may not be available at emergence sites (Assem, 1986). In such cases, natural selection would tend to favor a communication system that functions over distances greater than those associated with visual cues. With a life-span of only 5–30 days (Boyce, 1936) and only a narrow window of opportunity in which to reproduce, it is unlikely that adult *A. quadridentata* rely on random search to locate mates. It is more likely that they produce chemical signals, such as pheromones, to recruit mates over distances of several meters.

Evidence for sex pheromones has been found in hymenopteran parasitoids in the Aphelinidae, Chalcididae, Cynipidae, Pteromalidae, Scelionidae, Braconidae, Ichneumonidae (reviewed in Eller et al., 1984), Eulophidae (Finidori-Logli et al., 1996) and in the Trichogrammatidae (Pompanon et al., 1997). Typically, female parasitic wasps release pheromones to attract males (Boush and Baerwald, 1967; Cole, 1970; Vinson, 1972, 1978; Matthews, 1974; Obara and Kitano, 1974; Gordh and de Bach, 1978; Browning and Oatman, 1985; Kamano et al., 1989; Kainoh et al., 1991; Field and Keller, 1993; Pompanon et al., 1997), although either sex may signal, as males have been reported to form swarms that are attractive to females (Assem, 1986). In the presence of female pheromone extract or isolated pheromone, male wasps are stimulated to search and/or are attracted over a relatively short range of a few centimeters; there are very few demonstrations of long-range sex pheromones among parasitic wasps (Godfray, 1994).

Research objectives were to (1) obtain behavioral evidence that pheromones mediate mate-seeking behavior of *A. quadridentata*, (2) identify the pheromone(s), and (3) demonstrate behavioral activity of identified compounds in laboratory and field experiments.

METHODS AND MATERIALS

Laboratory Cultures

C. pomonella was reared on an artificial diet (modified from Brinton et al., 1969) under a 16L : 8D photoregime, 25°C, and 65% relative humidity. Mated female moths oviposited on sheets of wax paper. Each week, one sheet was

sent from the Agriculture and Agri-Food Canada Research Station in Summerland, British Columbia, to Simon Fraser University, where it was stored at ca. 4°C.

The colony of *A. quadridentata* was established and periodically augmented with specimens from the colony at Washington State University, Pullman, Washington. Adult *A. quadridentata* were maintained in mesh cages (30 × 30 × 45 cm) with a Plexiglass front under a 16L (27°C) : 8D (15°C) photoregime and 70% relative humidity. Insects were provided with a 10% honey–water solution, distilled water, and honey-covered filter paper ad libitum. Strips of wax paper with eggs of *C. pomonella*, at a ratio of 10 eggs per adult *A. quadridentata*, were placed into cages for ca. 24 hr. Sheets with parasitized eggs were transferred to covered plastic drinking cups (400 ml) with moist filter paper. Upon hatching, pairs of two neonates were transferred to, and encased within, smaller plastic cups (30 ml) provisioned with two cubes (ca. 1.5 × 1.5 × 1.5 cm) of artificial diet (Bio-Serv, Inc., Frenchtown, New Jersey). Male and female parasitoids were transferred to mesh cages as they emerged.

Behavioral Evidence for Presence of Sex Pheromones

Volatile Collection. To test the hypothesis that *A. quadridentata* release pheromones to attract mates, 12- to 168-hr-old unmated females and males were placed separately into horizontal cylindrical Pyrex glass aeration chambers (2.5 cm diam. × 18.5 cm long). A water aspirator drew humidified, charcoal-filtered air at a rate of 1.2 liters/min for five days through each chamber and corresponding glass column (14 cm × 1.3 cm OD) downwind filled with Porapak Q (50–80 mesh, Waters Associates, Inc., Milford, Massachusetts). Volatiles from female or male *A. quadridentata* were eluted from respective Porapak Q volatile traps, each with 2 ml of redistilled pentane.

Y-Tube Olfactometer Bioassay. Anemotactic responses of walking or flying *A. quadridentata* to odor sources were assessed at 25–27°C and 50–70% relative humidity in a vertical Y-shaped Pyrex glass olfactometer [stem: 20 cm long × 2.5 cm ID; side arms at 120°: 18 cm long; entrance hole for parasitoids (4 mm diam.) 5 cm above the rim of the stem]. Bioassays were conducted between 2 and 12 hr of the insects' photophase (8 AM–8 PM). Because *A. quadridentata* is positively phototactic, a single light source composed of tubes of fluorescent "daylight" (F40D H5b8; Osram Sylvania Ltd., Ontario, Canada) and "wide spectrum grow light" (F40GRO WS6 H568; Osram Sylvania Ltd., Ontario, Canada) at a 1 : 1 ratio (Shields, 1989) was centered above the vertical olfactometer. Radiometric irradiance at the top and base of the olfactometer was respectively 8.0 W/m^2 and 3.8 W/m^2 (Radiometer model IL1400A, International Light Inc.). Visual cues were standardized by enclosing the olfactometer on three sides with white poster board. Treatment and control odor sources were micropipetted onto What-

man No. 1 filter paper (4.25 cm) placed near the orifice of each side arm. For each replicate, a new (cleaned and oven-dried) Y-tube, insect, and filter paper were used, with test stimuli randomly assigned to side arms. Air drawn through the apparatus at 2.4–3.3 liters/min with a water aspirator carried volatiles from odor sources through the stem of the Y-tube. Thirty seconds after placement of stimuli, a parasitoid was released through the entrance hole of the olfactometer. Parasitoids that reached either the filter paper with the control solvent or the filter paper with the treatment solution within 15 minutes were classed as responders; all others were classed as nonresponders and were not included in statistical analyses.

Numbers of parasitoids responding to stimuli (>85%) were analyzed with the χ^2 goodness-of-fit test using Yates' correction for continuity ($\alpha = 0.05$) to determine whether observed frequencies deviated significantly from expected frequencies, under the null hypothesis that sampled *A. quadridentata* did not prefer either treatment or control odors (Zar, 1996).

Five experiments (Table 1) were conducted in Y-tube olfactometers to determine attractiveness of male or female *A. quadridentata*-produced volatiles. Experiments 1 and 2 tested response of males to two doses of female-produced volatiles to provide evidence for male-attractive pheromones. Experiment 3 determined whether mating of females altered the attractiveness of female-produced volatiles to males. To assess whether males produce pheromones, volatiles from male *A. quadridentata* were tested with female (experiment 4) and male (experiment 5) *A. quadridentata*. Except for experiment 3, pentane served as a solvent control because volatiles in treatment stimuli were dissolved in pentane.

Identification of Candidate Pheromones

Aliquots of ca. 1 female-hour-equivalent (FHE) of Porapak Q-captured volatiles were subjected to analysis by coupled gas chromatographic-electroantennographic detection (GC-EAD) (Arn et al., 1975), employing a Hewlett Packard (HP) 5890A gas chromatograph equipped with a fused silica column (30 m × 0.25 or 0.32 mm ID) coated with DB-5, DB-23, or DB-210 (J & W Scientific, Folsom, California 95630). For GC-EAD recordings, an antenna was gently pulled from an insect's head; the distal segment was removed, and it was then suspended between glass capillary electrodes filled with insect Ringer's solution (6.5 g/liter NaCl, 1.4 g/liter KCl, 0.12 g/liter $CaCl_2$, 0.1 g/liter $NaHCO_3$, 0.01 g/liter Na_2HPO_4, in 1 liter distilled H_2O). EAD-active compounds were identified using the following procedures: (1) full-scan electron-impact (EI) and chemical-ionization (isobutane) (CI) mass spectra (MS) obtained from a Varian Saturn II Ion Trap GC-MS and a HP 5985B GC-MS, respectively, each fitted with the DB-5 or DB-210 column referred to above; (2) retention index calculations (Dool and Kratz, 1963); and (3) microanalytical treatments (acetylation, hydrogenation and

TABLE 1. STIMULI TESTED IN Y-TUBE OLFACTOMETER EXPERIMENTS AND NUMBERS OF MALE OR FEMALE *A. quadridentata* (AQ) BIOASSAYED

	Test stimuli		Parasitoids tested	
Exp.	Treatment	Control[a]	Number[b]	Sex
1	1 female hour equivalent of female AQ volatiles in pentane	pentane	28 (28)	Male
2	10 female hour equivalents of female AQ volatiles in pentane	pentane	31 (30)	Male
3	10 female hour equivalents of mated female AQ volatiles	10 female hour equivalents of unmated female AQ volatiles	37 (37)	Male
4	1 male hour equivalent of male AQ volatiles in pentane	pentane	20 (19)	Female
5	1 male hour equivalent of male AQ volatiles in pentane	pentane	30 (27)	Male

[a]The volume of pentane in controls was identical to corresponding treatment volumes, ranging from 1–12 μL.
[b]Number of responding insects given in parentheses.

borohydride reactions) followed by renewed GC-MS. Differential diagnosis (GC and GC-MS) of volatiles from female and male *A. quadridentata* allowed determination of volatiles specifically produced by female or male parasitoids. Elucidations of chemical structures were confirmed by comparative GC, GC-MS, and GC-EAD analyses of insect-produced compounds and authentic standards. Purity and source of synthetic standards are as follows: (1) 3,7,11-trimethyl-6*E*,10-dodecadienal, 95% purity (synthesized by G.G.S. King, SFU); (2) (Z)-9-hexadecenal, 95% purity (obtained from Sigma Chemical Co., St. Louis, Missouri); and (3) (Z,Z)-9,12-octadecadienal, 97% purity, obtained by reducing linoleic acid (Sigma Chemical Co.) to alcohol, then oxidizing to aldehyde.

Testing of Candidate Pheromone Components

Laboratory Experiments. Response of *A. quadridentata* to synthetic female-specific candidate pheromone components {3,7,11-trimethyl-6*E*,10-dodecadienal (dihydrofarnesal), (Z)-9-hexadecenal [(Z)-9–16 : Ald] and (Z,Z)-9,12-octadecadienal [(Z,Z)-9,12–18 : Ald]} was tested in Y-tube olfactometer bioassays (Table 2). Experiments 6–12 tested response of males to these compounds singly at various doses. Experiment 13 tested response of female *A. quadridentata* to (Z,Z)-9,12–18 : Ald to ascertain whether this compound is a sex or aggregation pheromone. To determine potential synergism between

TABLE 2. STIMULI TESTED IN Y-TUBE OLFACTOMETER EXPERIMENTS AND NUMBERS OF MALE OR FEMALE *A. quadridentata* (AQ) BIOASSAYED

	Test stimuli		Parasitoids tested	
Exp.	Treatment[a]	Control[b]	Number[c]	Sex
6	10 ng dihydrofarnesal in hexane	hexane	15 (15)	Male
7	100 ng dihydrofarnesal in hexane	hexane	18 (17)	Male
8	10 ng (*Z*)-9-16:Ald in hexane	hexane	56 (51)	Male
9	100 ng (*Z*)-9-16:Ald in hexane	hexane	34 (29)	Male
10	1 ng (*Z,Z*)-9,12-18:Ald in hexane	hexane	34 (29)	Male
11	1 ng (*Z,Z*)-9,12-18:Ald in hexane	hexane	30 (28)	Male
12	10 ng (*Z,Z*)-9,12-18:Ald in hexane	hexane	49 (45)	Male
13	10 ng (*Z,Z*)-9,12-18:Ald in hexane	hexane	22 (22)	Female
14	10 ng dihydrofarnesal & 0.2 ng (*Z,Z*)-9,12-18:Ald in hexane	10 ng dihydrofarnesal in hexane	36 (35)	Male
15	10 ng (*Z,Z*)-9,12-18:Ald & 1 ng (*Z*)-9-16:Ald in hexane	10 ng (*Z,Z*)-9,12-18:Ald in hexane	22 (21)	Male
16	0.1 ng dihydrofarnesal, 0.1 ng (*Z*)-9-16:Ald, 0.1 ng (*Z,Z*)-9,12-18:Ald, all in hexane	0.1 ng (*Z,Z*)-9,12-18:Ald in hexane	41 (39)	Male
17	1 ng dihydrofarnesal, 1 ng (*Z*)-9-16:Ald, 1 ng (*Z,Z*)-9,12-18:Ald, all in hexane	1 ng (*Z,Z*)-9,12-18:Ald in hexane	36 (36)	Male
18	1 ng (*Z,Z*)-9,12-18:Ald in pentane and hexane	10 female hour equivalents of female AQ volatiles, containing 1 ng (*Z,Z*)-9,12-18:Ald in pentane and hexane	41 (39)	Male

[a]Compound abbreviations: dihydrofarnesal (3,7,11-trimethyl-6*E*,10-dodecadienal), (*Z*)-9-16:Ald ((*Z*)-9-hexadecenal), and (*Z,Z*)-9,12-18:Ald ((*Z,Z*)-9,12-octadecadienal).
[b]The volume of hexane or pentane in controls was identical to corresponding treatment volumes, ranging from 1-24 μL.
[c]Number of responding insects given in parentheses.

compounds, experiments 14–17 tested candidate pheromone components singly against binary (experiments 14, 15) and ternary (experiments 16, 17) combinations. Experiment 18 tested (*Z,Z*)-9,12–18 : Ald versus a volatile extract from female *A. quadridentata*, containing (*Z,Z*)-9,12–18 : Ald at the equivalent quantity. Unless otherwise specified, hexane was used as a solvent control because treatment stimuli were dissolved in hexane.

Field-Cage Experiments. To demonstrate pheromonal attractiveness of (*Z,Z*)-9,12–18 : Ald in the field, 48- to 168-hr-old laboratory-reared male *A. quadridentata* were released into screened field cages (3.60 × 3.60 × 3.60 m), each encompassing a single apple tree. Cages were located in the Entomology orchard of the Agriculture and Agri-Food Canada Research Station in Summerland, British

Columbia. Each tree received a treatment and a control trap, randomly assigned to respective north or south faces and suspended ca. 1.7 m above ground.

(Z,Z)-9,12–18 : Ald in hexane was micropipetted (10 μg: experiment 19; 100 μg: experiment 20) into gray rubber septa (The West Company, Lionville, Pennsylvania) just before the start of each replicate, while control septa received an equivalent volume of hexane. Lures were suspended in polyester (Commercial Plastics Inc., 3917 Grant Street, Burnaby, British Columbia) cylinders (ca. 12 cm ID × 12.5 cm wide) fitted with an equal-sized polyester insert covered with Stickem Special (Phero Tech Inc., Delta, British Columbia).

Between 9 and 32 laboratory-reared male *A. quadridentata* were released in each cage for each replicate from plastic cups (400 ml) with perforated lids, provisioned with honey-covered filter paper and a dental wick moistened with distilled water. Fifteen minutes after placement of release devices on the east side of each caged tree, ca. 0.5 m from the tree bole and the ground, experimental replicates were initiated by removing the cup lids.

The one-tailed Wilcoxon paired-sample test ($\alpha = 0.05$) was used to test the null hypothesis that numbers of male *A. quadridentata* caught in treatment traps were less than or equal to numbers caught in control traps, as the difference values obtained were roughly symmetrical about the median but could not be assumed to come from a normal distribution (Zar, 1996). Because proportions of male *A. quadridentata* caught in experiment 20 fit the normal approximation, data were analyzed by a one-tailed paired-sample t test ($\alpha = 0.05$) (Zar, 1996) to assess whether the difference in the proportion of insects caught in treatment traps was less than or equal to the proportion caught in control traps.

Field Experiment. A 10-replicate field experiment (experiment 21) tested attractiveness of (Z,Z)-9,12–18 : Ald in a feral population of *A. quadridentata* at Steptoe Butte State Park in Washington (lat. 47°09′N, long. 117°30′W) from June 26 to August 8, 1997. (Z,Z)-9,12–18 : Ald in hexane was micropipetted at four doses (0, 1, 10, or 100 μg) into rubber septa just before their placement into polyester traps. Position of treatments was completely randomized, with one trap hung in the upper third of each tree.

Results of experiment 21 were analyzed with a single-factor analysis of variance [model 1 (fixed-effects)] ($\alpha = 0.05$) under the null hypothesis that mean insect captures for all four treatments were equal. This was followed by the Tukey test ($\alpha = 0.05$) to determine which means were significantly different (Zar, 1996).

RESULTS

Behavioral Evidence for Presence of Sex Pheromones

A dose of 10, but not 1, FHE elicited significant behavioral response from male *A. quadridentata* (Figure 1, experiments 1 and 2). Volatiles (10 FHE) from

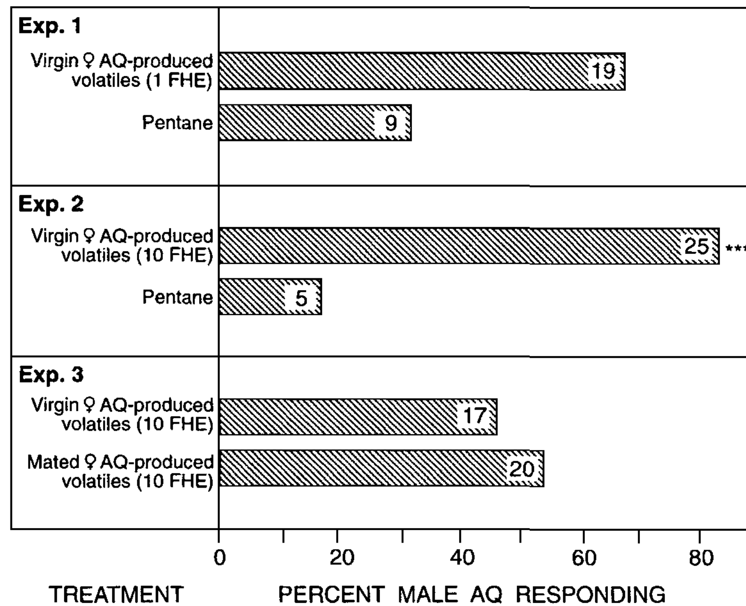

FIG. 1. Response of male *A. quadridentata* (AQ) to volatiles produced by virgin or mated female AQ (Table 1: experiments 1–3) or to solvent controls in a Y-tube olfactometer; FHE = female hour equivalent. Numbers of males responding to each stimulus given within bars for each experiment; bars with asterisks indicate a significant response to a particular treatment; χ^2 test with Yates' correction for continuity, treatment versus control; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

virgin and mated females were equally attractive to males (Figure 1, experiment 3). Wing fanning was induced in all three bioassays, and males were often arrested by the female-produced volatiles.

Male *A. quadridentata*-produced volatiles at 1 male-hour-equivalent (MHE) attracted male, but not female, *A. quadridentata* (Figure 2, experiments 4 and 5).

Identification of Candidate Pheromones

Coupled GC-EAD analyses of female *A. quadridentata*-produced volatiles, using an antenna of a male or female *A. quadridentata* as an electroantennographic detector, revealed three compounds that consistently elicited a response from male antennae (Figure 3). Of these, only the first compound elicited antennal response from female *A. quadridentata*.

Retention indices (RI) of EAD-active compound 1 on three fused silica

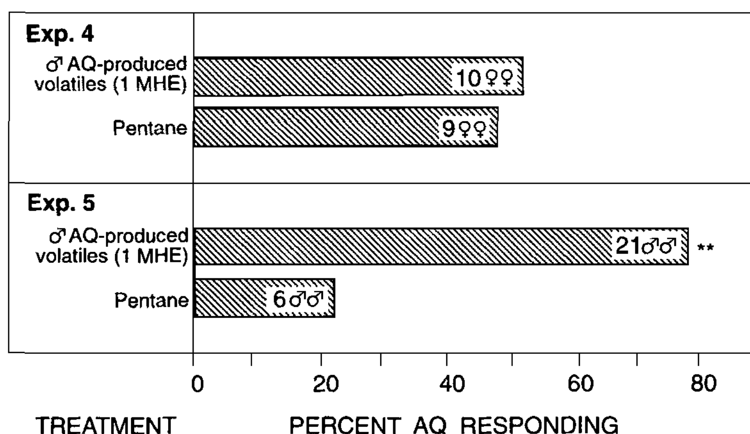

FIG. 2. Response of female (experiment 4) or male (experiment 5) *A. quadridentata* (AQ) to male AQ-produced volatiles; MHE = male hour equivalent (Table 1). Numbers of individuals responding to each stimulus given within bars. For each experiment, bars with asterisks indicate a significant response to a particular treatment; χ^2 test with Yates' correction for continuity, treatment versus control; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

columns (DB-23: RI = 2078; DB-5: RI = 1620; DB-210: RI = 1929) were indicative of an aldehyde functionality. Both reduction with $NaBH_4$ and unsuccessful acetylation with pyridine and acetic anhydride of the HPLC-isolated compound 1 supported an aldehyde functionality. Mass spectra of reduced compound 1 and of 3,7,11-trimethyl-2*E*,6*E*,10-dodecatrien-1-ol (*E*,*E*-farnesol) were similar, except fragmentation ions of compound 1 indicated two, instead of three, unsaturations of the molecule. Selective hydrogenation (House, 1972) of 3,7,11-trimethyl-2*E*,6*E*,10-dodecatrienal (*E*,*E*-farnesal) resulted in 3,7,11-trimethyl-6*E*,10-dodecadienal (dihydrofarnesal), which had mass spectral [EI-MS m/z (relative intensity): 222 (M, 2), 205 (6), 189 (4), 179 (35), 161 (27), 149 (10), 135 (9), 123 (32), 121 (11), 109 (38), 107 (16), 95 (22), 93 (13), 81 (29), 79 (11), 70 (14), 69 (67), 68 (20), 67 (43), 55 (11), 53 (13), 43 (16), 41 (100)], retention, and electrophysiological characteristics on the three columns mentioned above consistent with those of EAD-active compound 1.

Quantities of EAD-active compound 2 were insufficient to obtain a mass spectrum. Retention indices of this compound on DB-5, DB-23, and DB-210 columns (1805, 2280, and 2153, respectively) were indicative of a hexadecenal. Five hexadecenals [(*Z*)-6-, (*Z*)-7-, (*Z*)-8-, (*Z*)-9-, and (*E*)-13-hexadecenal) had retention times similar to that of the unknown. Of these, only (*Z*)-9-hexadecenal cochromatographed with EAD-active compound 2 on each of the three DB-columns and elicited comparable EAD-activity.

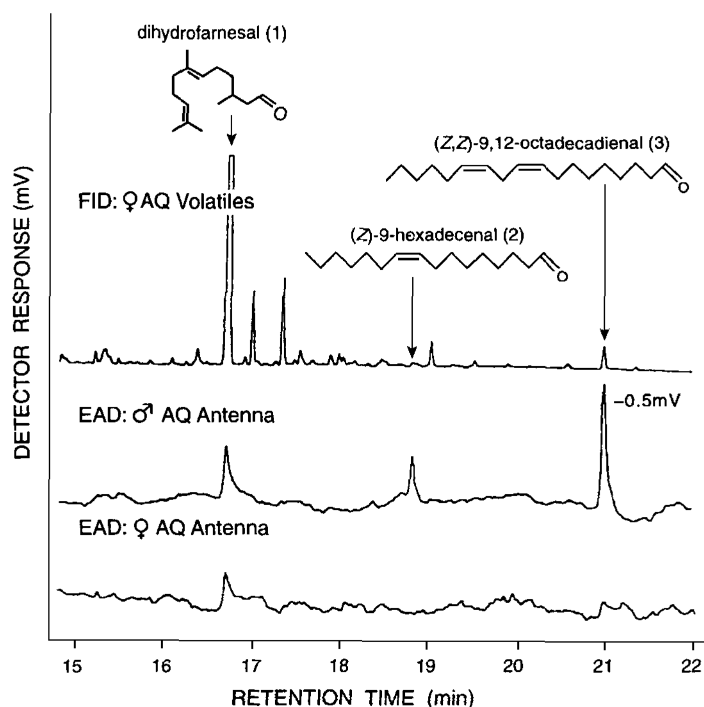

FIG. 3. Flame ionization detector (FID) and electroantennographic detector [EAD: *A. quadridentata* (AQ) antenna] responses to aliquots of 1 FHE of volatiles from female AQ. Chromatography: Hewlett Packard 5890A equipped with a DB-5 coated column (30 m × 0.25 mm ID); linear flow velocity of carrier gas: 35 cm/sec; injector and FID detector temperature: 240°C; temperature program: 1 min at 50°C, 10°C/min to 240°C.

The mass spectrum of EAD-active compound 3, with strong molecular ion *m/z* 264 and evidence for two double bonds (*m/z* 67, 81, and 95), suggested an octadecadienal (Heller and Milne, 1978). With (*Z,Z*)-9,12-octadecadienoic (linoleic) acid as a conceivable precursor, EAD-active compound 3 was hypothesized, and through comparative GC-MS and GC-EAD with an authentic standard, confirmed to be (*Z,Z*)-9,12-octadecadienal. Geometrical isomers of (*Z,Z*)-9,12-octadecadienal, obtained by treating isomeric 9,12-octadecadienoic acid methyl ester (Sigma Chemical Co.) with $LiAlH_4$ and then with pyridinium chlorochromate, had different retention times than compound 3 on the three DB columns.

Testing of Candidate Pheromone Components

At a 10- or 100-ng dose, dihydrofarnesal (Figure 4, experiments 6 and 7) or (Z)-9–16 : Ald (Figure 4, experiments 8 and 9) singly were no more attrac-

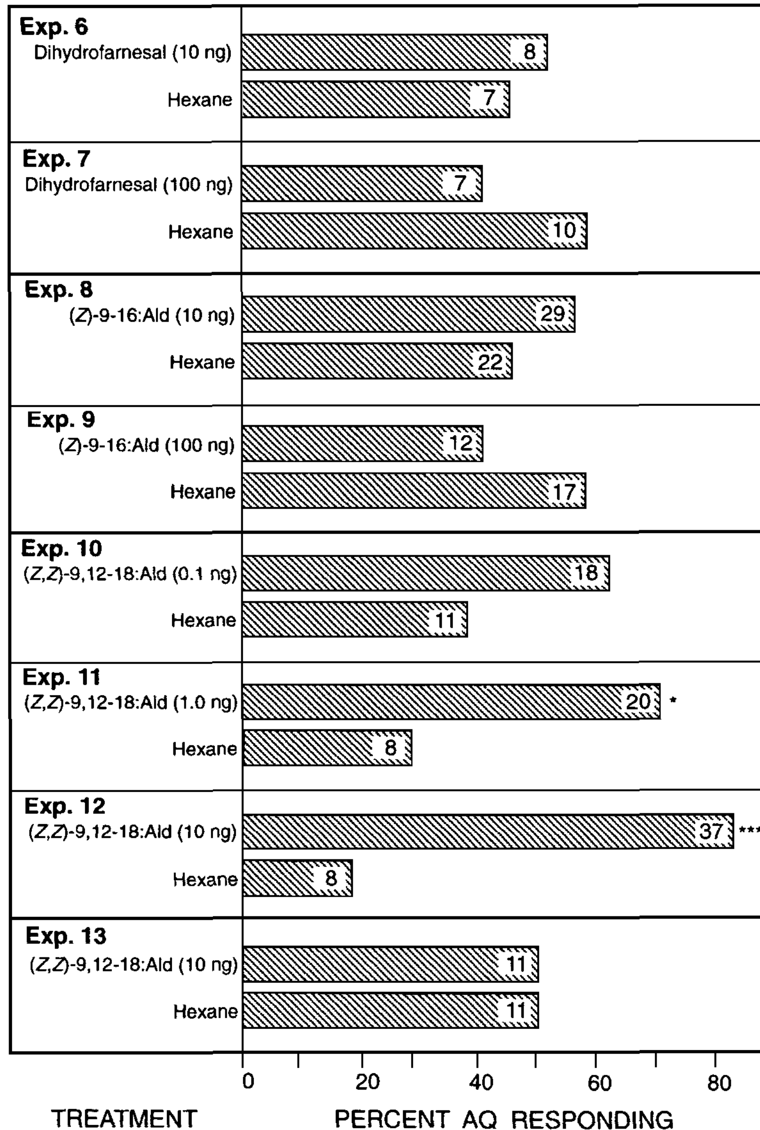

FIG. 4. Response of male (experiments 6–12) or female (experiment 13) *A. quadridentata* (AQ) to synthetic candidate pheromone components (Table 2). Numbers of insects responding to each stimulus given within bars. Asterisks indicate a significant response to a particular treatment; χ^2 test with Yates' correction for continuity, treatment versus control; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

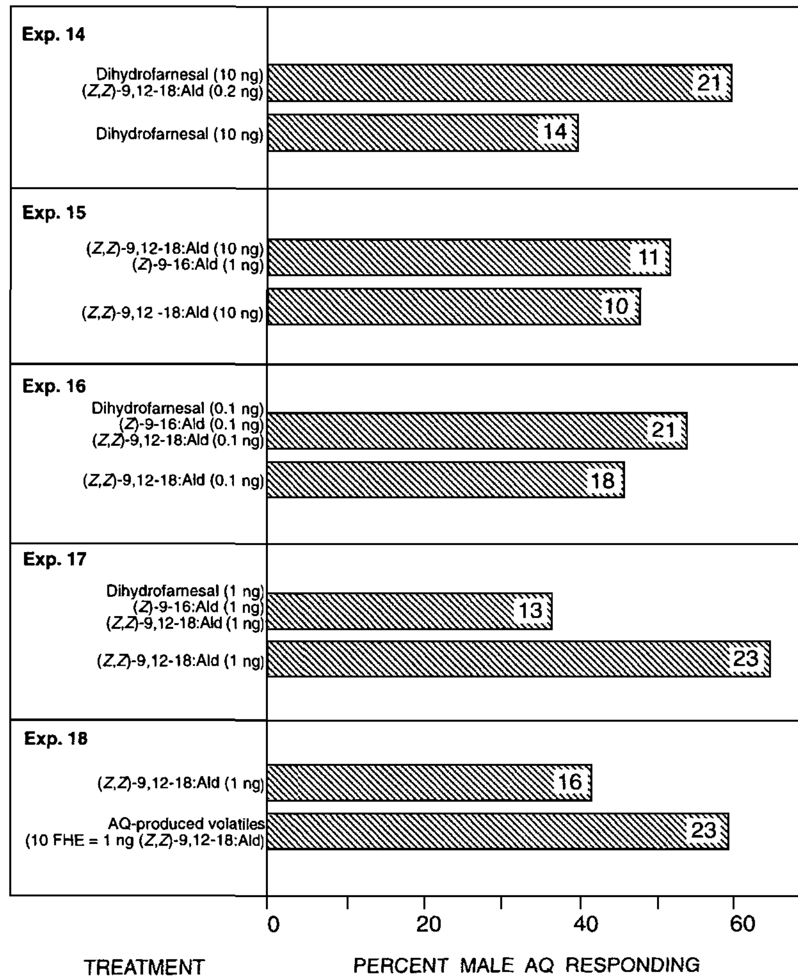

FIG. 5. Response of male *A. quadridentata* (AQ) to candidate pheromone components singly and in combinations (Table 2). Numbers of insects responding to each stimulus given within bars. Asterisks indicate a significant response to a particular treatment; χ^2 test with Yates' correction for continuity, treatment versus control; there was no statistically significant response to treatments in experiments 14–18.

tive to male *A. quadridentata* than a hexane control. Attractiveness of (*Z,Z*)-9,12–18 : Ald increased dose dependently (Figure 4, experiments 10–12). In contrast, female *A. quadridentata* were not attracted to (*Z,Z*)-9,12–18 : Ald (Figure 4, experiment 13).

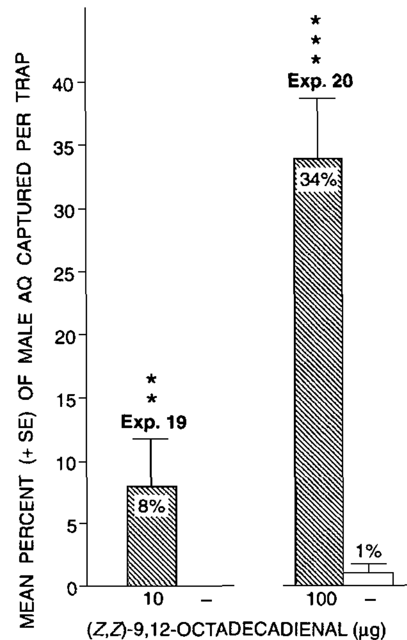

FIG. 6. Mean percent capture of released male *A. quadridentata* (AQ) in polyester cylinder traps baited with 10 μg (experiment 19: $N = 8$) and 100 μg (experiment 20: $N = 13$) of (Z,Z)-9,12-octadecadienal in field-cage bioassays, Summerland, British Columbia. For each experiment, bars with asterisks indicate a significant response to a particular treatment; experiment 19: one-tailed Wilcoxon paired-sample test ($\alpha = 0.05$); experiment 20: one-tailed paired-sample t test on proportions ($\alpha = 0.05$), treatment versus control; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

There was no synergistic effect between (Z,Z)-9,12–18 : Ald and dihydrofarnesal (experiment 14) nor (Z)-9–16 : Ald (experiment 15) (Figure 5). A ternary blend of synthetic (Z,Z)-9,12–18 : Ald, (Z)-9–16 : Ald, and dihydrofarnesal at ratios of 1 : 1 : 1 ng or 0.1 : 0.1 : 0.1 ng was as attractive as (Z,Z)-9,12–18 : Ald singly at the equivalent quantity (Figure 5, experiments 16 and 17). Synthetic (Z,Z)-9,12–18 : Ald and female *A. quadridentata*-produced volatiles, containing the equivalent amount of (Z,Z)-9,12–18 : Ald, were equally attractive to male *A. quadridentata* (Figure 5, experiment 18).

In field-cage bioassays, (Z,Z)-9,12–18 : Ald at 10 μg (Figure 6, experiment 19) or 100 μg (experiment 20) captured a significantly higher number of male *A. quadridentata* than did solvent controls. Captures of male *A. quadridentata* in the field experiment increased with increasing doses of (Z,Z)-9,12–18 : Ald (Figure 7, experiment 21).

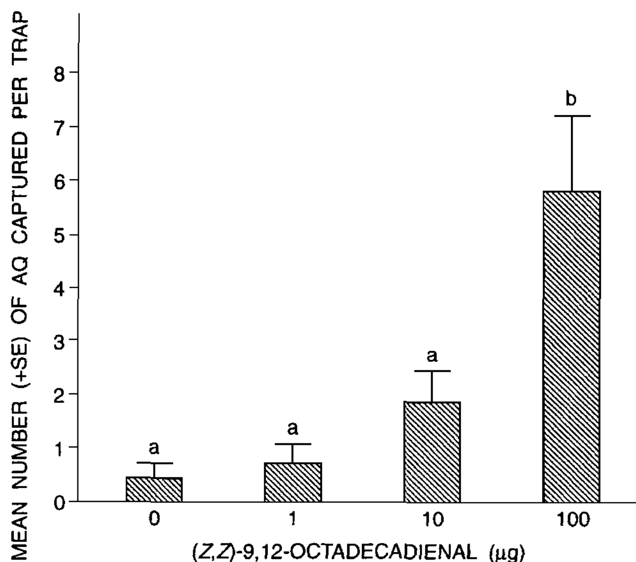

FIG. 7. Mean numbers of *A. quadridentata* (AQ) captured in polyester cylinder traps baited with (Z,Z)-9,12-octadecadienal at increasing doses (experiment 21). Steptoe Butte State Park, Washington, USA; $N = 10$. Bars with the same letter superscript are not significantly different, Tukey's test, $\alpha = 0.05$.

DISCUSSION

Evidence that (Z,Z)-9,12–18 : Ald is a pheromone component of female *A. quadridentata* includes the following: (1) (Z,Z)-9,12–18 : Ald was identified in the effluvia of female but not male *A. quadridentata*; (2) synthetic (Z,Z)-9,12–18 : Ald elicited strong antennal responses from male *A. quadridentata* in GC-EAD recordings (Figure 3); (3) it induced amplified antennal movement, wing vibrations, and accelerated walking in male *A. quadridentata*; and (4) it attracted male *A. quadridentata* in Y-tube olfactometer bioassays (Figure 4, experiments 10–12), field-cage bioassays (Figure 6, experiments 19 and 20), and in a field experiment (Figure 7, experiment 21).

Although eliciting antennal activity, neither (Z)-9–16 : Ald nor dihydrofarnesal singly were attractive to male *A. quadridentata* (Figure 4, experiments 6–9), nor did they enhance the attractiveness of (Z,Z)-9,12–18 : Ald (Figure 5, experiments 14–17). Electrophysiological activity of (Z)-9–16 : Ald may be interpreted as olfactory recognition of a pheromone component from a congener. In *A. recticulatus* Watanabe, (Z)-9–16 : Ald serves as a contact pheromone that may provide information about potential mates at close range (Kainoh et al.,

1991). Whether or not (Z)-9–16 : Ald plays a similar role in *A. quadridentata* remains to be investigated.

Lack of behavioral activity from dihydrofarnesal may be attributed to one or more of several factors: (1) dihydrofarnesal is also produced by male parasitoids and may indeed not be a pheromone component of female *A. quadridentata*; (2) female *A. quadridentata* may stereoselectively biosynthesize only one enantiomer (or a specific ratio of enantiomers) of dihydrofarnesal, and response by male *A. quadridentata* may have been inhibited by the unnatural enantiomer (or incorrect ratio) in enantiomeric synthetic dihydrofarnesal that was bioassayed (Figure 4, experiments 6 and 7); (3) because dihydrofarnesal was the only compound in GC-EAD recordings that also elicited responses from female antennae (Figure 3), this compound may mediate chemical communication between female parasitoids rather than attracting mates. Similar attractiveness of synthetic (Z,Z)-9,12–18 : Ald and of volatiles from female *A. quadridentata*, containing the equivalent amount of (Z,Z)-9,12–18 : Ald (Figure 5, experiment 18), suggests that female *A. quadridentata* may indeed employ (Z,Z)-9,12–18 : Ald as a single-component sex pheromone to attract mates.

Comparable responses of male *A. quadridentata* to volatiles from virgin and mated females (Figure 1, experiment 3) suggest that mating does not affect pheromone production in females, which is consistent with observations of repetitive matings by female and male *A. quadridentata*. When male parasitoids perceive the female's pheromone, they increase antennal movement, vibrate their wings, and follow the female in an accelerated manner. Pulling air over their body through wing vibrations may help males orient toward the pheromonal source (Vinson, 1972). Sound associated with wing vibrations likely resonates from the chitinous wall of the thorax (Assem and Putters, 1980) and seems to be common during courtship in parasitic wasps (Miller and Tsao, 1974; Assem and Putters, 1980; Assem, 1986; Sivinski and Webb, 1989; Field and Keller, 1993). Wing vibration and sound production by male parasitoids appear to occur primarily in response to female-produced sex pheromones and in some species are necessary for the female to permit continuation of courtship (Obara and Kitano, 1974; Assem and Putters, 1980).

The role of the male-produced and male-attractive pheromone is not clear, but several interpretations are conceivable: (1) males responding to male-produced volatiles (Figure 2, experiment 5) and detecting other males in search of females may enhance their probability of encountering females; (2) males attracted to low levels of male-produced pheromone may form leks, as found in other parasitoids (Assem, 1986), collectively produce more pheromone and thus become more attractive to foraging females; or (3) they may release pheromone which, together with wing vibration, induces mating receptivity in females rather than directional responses (Assem and Putters, 1980; Assem, 1986) (Figure 2, experiment 4).

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THE SESTERTERPENE VARIABILIN AS A FISH-PREDATION DETERRENT IN THE WESTERN ATLANTIC SPONGE *Ircinia strobilina*

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Abstract—The furanosesterterpene variabilin was identified in an unpalatable crude extract and proved to be a feeding deterrent when offered at 0.23% of artificial diet dry weight to reef fishes in field assays. The icthyodeterrent property of variabilin was expressed when food pellets for the palatability assays were made with calcium alginate, but not carrageenan. Variabilin probably failed to show deterrent activity in the carageenan matrix because of decomposition during the necessary heat treatment.

Key Words—*Ircinia strobilina*, Thorectidae, Dictyoceratida, sponge, Brazil, southwest Atlantic, variabilin, furanosesterterpenes, feeding deterrency, marine chemical ecology.

INTRODUCTION

Sponges are abundant and ecologically important members of many communities, including those associated with coral reefs. They are efficient filter feeders, provide shelter for invertebrates and fishes, harbor symbionts, compete with other sessile invertebrates for space, and can cause bioerosion on coral reefs (Pawlik et al., 1995).

Recent investigation of Caribbean sponges from various habitats suggested

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that their distribution and abundance is determined by predation and not by other physical or biological factors. All Demospongiae seem to be potentially attractive prey to fishes, but when these sponges are made equally available to reef fishes, only mangrove or cryptic species are quickly consumed. These observations can explain, in part, why many mangrove or cryptic species of Demospongiae are absent from reefs (Dunlap and Pawlik, 1996).

Bioassays with sponge crude extracts, incorporated into artificial food and offered to cooccurring fishes, indicated that only those sponges avoided by potential predators have unpalatable chemicals in their tissues (McClintock et al., 1997; Pawlik et al., 1995). One such sponge group is the Caribbean Thorectidae (Dictyoceratida). However, the metabolites responsible for predator aversion are still unknown (Pawlik, 1993; Pawlik et al., 1995). Sponges are a rich source of complex and unique chemicals (Faulkner, 1998), with a variety of biological activities, and some have potential medical applications (Bongiorni and Pietra, 1996; Carté, 1996; Faulkner, 1995).

There is increasing interest in the icthyodeterrent properties of sponge compounds, but evidence of ecological activities for pure sponge metabolites are few or variable depending on the experimental design used (Pawlik, 1993; Pennings et al., 1994).

Ircinia strobilina (Thorectidae, Dictyoceratida) is an unpalatable Caribbean species studied previously (Pawlik et al., 1995), and it is the most abundant sponge growing exposed in the presence of a large number of predatory fishes on the characteristic Abrolhos mushroom-like reefs, now preserved in the south of Bahia State, Brazil.

The objectives of the present study were to verify that Brazilian *I. strobilina* is chemically defended against fish predators, and to identify the metabolite(s) responsible for such defense.

METHODS AND MATERIALS

Sponge Collection. I. strobilina Lammarck 1816 (Thorectidae, Dictyoceratida) was collected at 10 m depth by scuba diving in Timbebas reef, National Marine Park of Abrolhos (17°30′S; 39°00′W; Bahia State, Brazil) during January 1996. *I. strobilina* is one of the most abundant sponges growing exposed on the characteristic Abrolhos mushroom-like reefs (chapeirões). It is a massive, elastic, but relatively tough and difficult to tear, brownish gray sponge, with a skeleton formed by spongin fibers of variable thickness and devoid of spicules. Voucher specimens were deposited in the sponge collection of Universidade Federal Fluminense (ABR9602). Samples were stored in ethanol (EtOH) until laboratory analyses.

Crude Extract and Variabilin. The EtOH was removed and the sponge tissue (850 g of dried material after extraction) was further extracted with a mixture

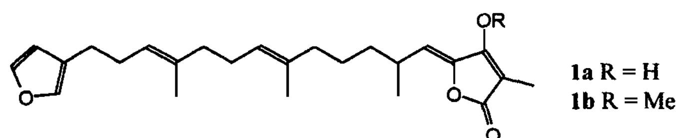

FIG. 1. Chemical structures of variabilin and its methoxy derivative.

of methanol–methylene chloride ($MeOH/CH_2Cl_2$, 6 : 4). After evaporation under vacuum, the combined extracts were partitioned between hexane–methanol, ethyl acetate–water and *n*-butanol–water; furnishing hexane (4.73 g), ethyl acetate (EtOAc) (8.25 g), *n*-butanol (4.20 g), and water (71.58 g) fractions, respectively. Vacuum and flash chromatography of part of the resulting EtOAc fraction (2.63 g) over silica gel with a hexane–ethyl acetate gradient, yielded 107.0 mg of the sesterterpene **1a** (Figure 1). Thirty milligrams of **1a** was treated with diazomethane (CH_2N_2) in diethyl ether and purified by semipreparative HPLC (silica gel, hexane–EtOAc, 4 : 1) to give 6.0 mg of **1b**. Variabilin (**1a**) and its methoxy derivative (**1b**) were identified by comparison of MS, UV, ^1H, and ^{13}C NMR data with literature values (Barrow et al., 1988a,b). During this work we observed that pure variabilin was readily decomposed when exposed to air or light or in chloroform ($CHCl_3$) solution.

Palatability Assays. I. strobilina crude extract was incorporated at its natural concentration into carrageenan-based and calcium alginate-based artificial diets, and pure variabilin (**1a**) was added into calcium alginate diets as described below. During the experiments several common tropical fishes well known to occur in the studied area (Nunan, 1992) were observed feeding on test strips (e.g., belonging to the Families Haemulidae, Labridae, Tetraodontidae, Balistidae, Sparidae, and Ostraciidae). Field assays and underwater observations were performed with aid of a portable surface supply diving apparatus. The Wilcoxon paired-sample test [one-tailed (Zar, 1984)] was used to analyze the results (Pawlik and Fenical, 1992).

Carrageenan food strips were prepared by an established methodology (Fenical and Pawlik, 1991; Pawlik and Fenical, 1992), involving combination of 2.5 g carrageenan (Sigma C-1013 type 1), 60 ml water and 20 ml commercial tunafish puree packed in oil. *I. strobilina* crude extracts were incorporated in treated food strips at the same concentration occurring in the living organisms (278.5 mg to a final volume of 60 cm^3). For each experiment, 20 treated and 20 control strips (1.0 × 0.6 × 5.0 cm each) were prepared and arranged in pairs and attached to 20 ropes. The ropes were anchored slightly above the bottom at Tartaruga Beach, Armacão dos Búzios, Rio de Janeiro State, Brazil (22°45′S; 41°51′W). Within 3 hr the ropes were retrieved and the amount of each strip eaten was measured.

Crude extract (190.0 mg) and pure variabilin (**1a**, 6.9 mg) were reconstituted in a matrix of calcium alginate (final volume of 40 cm^3) and assayed at Tartaruga Beach. The alginate strips were made by combining 1 g sodium alginate, 34 ml distilled water, and 6 ml commercial tunafish puree (canned tuna in oil, pureed in a minimal volume of distilled water in a blender). The extract or pure compound was added to this mixture without any solvent. The mixture was vigorously stirred and poured into acrylic molds crossed by lengths of cotton string protruded from the ends of the molds. The mixture was hardened by exposure to an aqueous solution of calcium chloride; exposure was enhanced by punching channels into the hardening mixture with a hypodermic needle. Strips measuring 1.0 × 0.4 × 5.0 cm were sliced with a blade and removed from the molds. Subsequent procedures were identical to those described for the carrageenan strips (Pawlik and Fenical, 1992).

Variabilin was also added to a small amount of an artificial diet prepared with carrageenan matrix and then extracted with CH_2Cl_2. The resulting material was compared with pure variabilin by TLC in silica gel (hexane–EtOAc, 1 : 1), revealing that the sesterterpene had decomposed.

RESULTS

I. strobilina crude extract was avoided by reef fishes when incorporated into carrageenan or calcium alginate strips at its natural concentration (Figures 2 and 4, P = 0.0030 and P = 0.0061, respectively). Purification of the crude extract by partition with different solvents and chromatographies afforded the

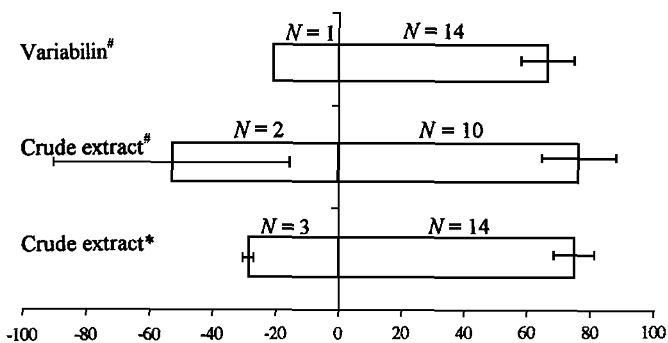

FIG. 2. Mean paired differences in percent eaten between control and treated strips containing *I. strobilina* crude extract and pure variabilin. Vertical lines through each histogram indicate SE. *Carrageenan-based pellets; #Alginate-based pellets; N = number of pairs with negative or positive (control pellets less or more consumed than treatment pellets, respectively) differences.

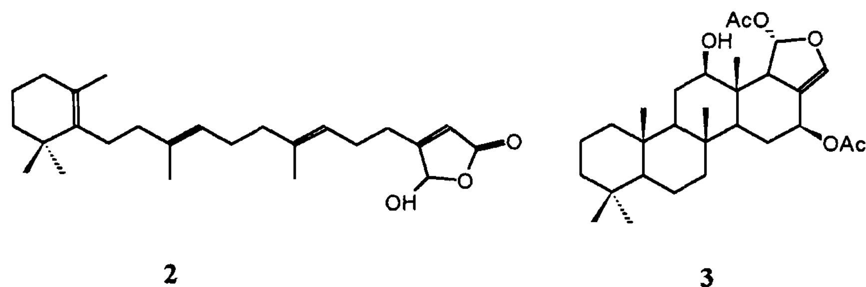

FIG. 3. Chemical structures of luffariellolide (**2**) and heteronemin (**3**).

known sesterterpene variabilin (**1a**, Figure 1). Variabilin was identified by spectroscopic methods and by preparation of its methoxy derivative **1b** (Figure 1) (Barrow et al., 1988a,b). It was observed that pure variabilin was decomposed during laboratory procedures or when incorporated into carrageenan-based diet. In order to avoid the heating and consequent degradation of variabilin, palatability assays were performed with calcium alginate-based artificial food (Epifanio et al., 1999). These baits, containing 0.23% of variabilin on a dry weight basis, were avoided by fishes when compared to controls ($P = 0.0001$, Figures 2 and 4).

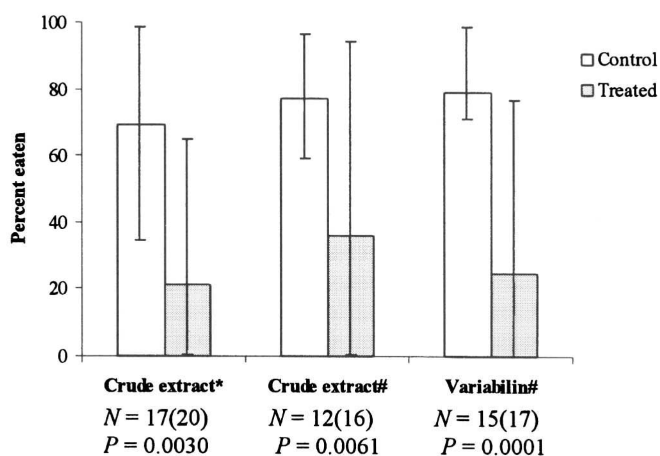

FIG. 4. Consumption by coocurring fishes of paired bait-strips with (treated) and without (control) *I. strobilina* crude extracts or variabilin. *Carrageenan-based strips. #Calcium alginate-based strips. Error bars are ± SD (data were transformed to SQRT arcsin proportion; SD was calculated and retransformed before plotting). N = no. of paired treated and control strips used in statistical analysis (in parentheses = no. of pairs retrieved of 20 deployed). P = probability calculated by the Wilcoxon paired-sample test, one-tailed.

DISCUSSION

Ecologically relevant palatability assays developed during the last decade have used various techniques to test natural volumetric or dry weight concentrations of secondary metabolites in the field or in aquaria. In general, metabolites are incorporated into a flavored polysaccharide matrix and offered to potential consumers (Hay et al., 1998). Depending on their polysaccharide nature, the matrices can be hardened by heating or cation exchange. Such experiments have identified a large number of crude sponge extracts that are avoided by cooccurring fishes in aquaria (McClintock et al., 1997; Pawlik et al., 1995). Some pure compounds isolated from the bioactive mixtures, however, had no or variable effects, depending upon how they were tested (Pennings et al., 1994).

Our results show that a crude extract of the Dyctioceratida sponge *I. strobilina* collected in Brazil is unpalatable to a natural assemblage of reef fishes in the field. Chemical investigation of a specimen from the Florida Keys showed that *I. strobilina* produces variabilin and its isomer strobilin in a ratio of 3 : 1 (Rothberg and Shubiak, 1975). We found that variabilin (**1a**), the only sesterterpene isolated from the Brazilian population, incorporated into an alginate matrix at a concentration of 0.23% of artificial diet dry weight reduced consumption of baits relative to controls. Because variabilin was present in other fractions of the crude extract and decomposed during purification procedures, we could not estimate the natural concentration of the metabolite in sponge tissue. The concentration used, however, was lower than that used in other studies of sponge metabolites (Thompson et al., 1985; Pennings et al., 1994). The observation of feeding deterrence due to variabilin was only possible because a nondestructive method was used to prepare the artificial diets. With the carrageenan matrix, which needs heat to harden, variabilin decomposed. Possible decomposition of active compounds may explain in part why only few sponge metabolites have been recognized to protect sponges from predators and why others have variable effects, although many crude extracts have proven to be active.

Other tropical western Atlantic sponges also contain predation-deterring metabolites in their tissues when crude extracts or fractions incorporated into calcium alginate pellets were tested in aquaria (McClintock et al., 1997; Pawlik et al., 1995). Sponges of the order Dyctioceratida are known to produce sesqui-, di-, or sesterterpenes. Most of the compounds have furanic, tetronic acid, or related moieties, such as [variabilin (**1a**) luffariellolide (**2**) and heteronemin (**3**) (Figure 3) (Faulkner, 1998). However, sesterterpenes **2** and **3**, isolated from the Guam sponges *Hyrtios* spp., were palatable to predators in the field when incorporated into carrageenan or agar-based baits (Pennings et al., 1994). Heteronemin had deterrent properties only if applied over squid strips at a concentration of 1–1.5% (Rogers and Paul, 1991). As secondary metabolites are usually more effective in low-quality diets (Chanas and Pawlik, 1996), it was proposed that the

use of squid strips could have influenced these results (Rogers and Paul, 1991). On the other hand, the same metabolite was incorporated into low-quality carrageenan based tubifex diet and did not deter feeding, even at a concentration of 5% (Pennings et al., 1994). Whatever the diet quality, when the carrageenan or agar-agar baits are heated during preparation, probably heteronemin is decomposed and the feeding deterrence lost.

It is known that when some metabolites obtained from natural sources are purified, they easily decompose because the living organism or its crude extract contain natural stabilizers [e.g., antioxidizers (Epifanio et al., 1998)]. In comparison to metabolites isolated from marine natural sources, those from sponges are usually complex, difficult to isolate, and subject to decomposition. If a feeding deterrence is attributed to a given metabolite, it must be supposed also that the metabolite is reactive in the biological system, for example, by binding to enzymes that control physiological processes as one possible mechanism. Assuming a relationship between biological activity (reactivity) and susceptibility to decomposition, bioassays must be carefully designed in order to elucidate chemically mediated processes, taking into account not only the different aspects of feeding, but also avoiding the loss of labile metabolites.

Several furanic, tetronic acid, or related compounds containing derived forms of 1,4-dialdehydes or 1,4-diketones are reactive compounds that can suffer decomposition by heating or contact with trace amounts of acid. Terpenoids containing such moieties in their structures were proposed to act as defensive chemicals in Doridacea nudibranchs (Cimino et al., 1988), in octocorals (Epifanio et al., 1999), and in marine algae (Paul and Fenical, 1987). They may comprise a general class of feeding deterrents against fishes that, due to the similar reactivity, possess the same but still unknown mechanism of action.

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CHEMICAL DEFENSES AGAINST FISH PREDATION IN THREE BRAZILIAN OCTOCORALS: 11β,12β-EPOXYPUKALIDE AS A FEEDING DETERRENT IN *Phyllogorgia dilatata*

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Abstract—The feeding-deterrence properties of crude extracts of three Brazilian octocoral species, *Neospongodes atlantica* Kükenthal (Alcyonacea, Nephtheidae), *Plexaurella regia* Castro (Gorgonacea, Plexauridae), and *Phyllogorgia dilatata* Esper (Gorgonacea, Gorgoniidae), were investigated. All the extracts were incorporated into food strips at the concentrations occurring in the living organisms. Crude extract and its ethyl acetate fraction obtained from *P. dilatata* collected in Armação dos Búzios (Rio de Janeiro State), when incorporated into artificial diets and tested in the habitat of origin, reduced consumption of food strips by fishes, relative to controls. Crude extracts from two octocoral species collected at the National Marine Park of Abrolhos (Bahia State), *N. atlantica* and *P. regia*, had no apparent feeding-deterrence properties; in fact, they seemed to stimulate feeding. Bioassay-guided fractionation of the bioactive *P. dilatata* crude extract revealed that the deterrent property was restricted to a medium polarity fraction. Field palatability experiments with two pure compounds isolated from this fraction revealed that the furanocembranolide 11β,12β-epoxypukalide is a potent feeding deterrent produced by *P. dilatata* against fish. Apparently, furanocembranolides are a particular class of compounds with feeding deterrent properties, protecting some octocorals from potential fish predator species in both tropical and temperate environments.

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Key Words—*Phyllogorgia dilatata*, *Neospongodes atlantica*, *Plexaurella regia*, Octocorallia, chemical ecology, feeding deterrent, field palatability assay, epoxypukalide, southwest Atlantic, Brazil.

INTRODUCTION

Secondary metabolites produced by living organisms presumably have adaptive value that favors their evolution. Recent research has provided better understanding of a variety of functions of these compounds as chemical mediators in different intra- and interspecific interactions of marine communities (Hay and Fenical, 1997; Paul, 1992; Pawlik, 1993).

Marine octocorals (Phylum Cnidaria) are a prolific source of biologically active and structurally unique compounds isolated and described by natural-products chemists (Faulkner, 1998, and previous reviews cited therein). Although these metabolites have been assumed important for the success of octocorals in the marine environment, only in this decade have their real ecological functions been experimentally tested (Paul, 1992; Pawlik, 1993). Among properties attributed to these compounds, a defensive role against predation has been the most analyzed, in part due to the low predation rates found in these sessile and soft-bodied animals. Living in habitats characterized by high levels of predation and nutrient scarcity (Paul, 1992), octocorals seem to be free from predation with the exception of some specialized consumers [e.g., polychaetes (Vreeland and Lasker, 1989), mollusks (Paul, 1992), and butterflyfishes (Lasker, 1985)]. Studies carried out to verify the efficacy of octocoral metabolites as feeding deterrents have been conducted in Caribbean (Cronin et al., 1995; Fenical and Pawlik, 1991; Gerhart, 1984; Harvell et al., 1988; Pawlik and Fenical, 1989, 1992; Pawlik et al., 1987), Pacific (Van Alstyne et al., 1994; Wylie and Paul, 1989), and western North Atlantic waters (Gerhart and Coll, 1993), revealing that most of these invertebrates produce defensive chemicals against natural predators.

The extensive Brazilian coast (more than 7500 km) is rich in marine organisms, but assessments of their natural products have been rare and restricted to very few publications (Epifanio et al., 1998, 1999; Martins and Epifanio, 1998). The purpose of the present study was to evaluate for the first time the feeding deterrence properties of metabolites produced by three endemic Brazilian octocorals, *Neospongodes atlantica* (Nephtheidae), *Plexaurella regia* (Plexauridae), and *Phyllogorgia dilatata* (Gorgoniidae), against fishes in their natural habitat.

METHODS AND MATERIALS

Octocoral Collection. Colonies of *N. atlantica* and *P. regia* were collected on Timbebas reefs, National Marine Park of Abrolhos, Bahia State, Brazil

(17°30′S; 39°00′W) in January 1996 by scuba diving at about 10 m depth. The gorgonian *P. dilatata* was collected off Tartaruga beach, Armação dos Búzios, Rio de Janeiro State, Brazil (22°45′S; 41°51′W) in December 1995 by free diving between 3 and 5 m depth.

Phyllogorgia dilatata Esper, 1806 (Gorgonacea, Gorgoniidae), forms fanlike colonies reaching 65 cm high and 45 cm wide. This species is found only in Brazil, occurring from the coast of Ceará State to the north of Rio de Janeiro State, including Fernando de Noronha, Trindade and Atol das Rocas islands (Castro, 1990).

Plexaurella regia Castro, 1989 (Gorgonacea, Plexauridae) forms ramified cylindrical colonies up to 60 cm high. It generally occurs in shallow, well-illuminated waters, being common and only found on the top of *chapeirões*, characteristic Abrolhos mushroom-like reefs, south of Bahia State (Castro, 1989).

Neospongodes atlantica Kükenthal, 1903 (Alcyonacea, Nephtheidae) is the only soft coral species found on the Brazilian coast, and it is also endemic. The colonies are ramified, flexible, and reach 25 cm in height. This octocoral occurs from the coast of Rio Grande do Norte State to Bahia State. In general, it is found close to the base of reef slopes, where sediments accumulate (Castro, 1990).

Colonies of *N. atlantica* were stored in ethanol. *P. regia* and *P. dilatata* colonies were air-dried for 2 hr and kept frozen until laboratory analyses.

Extracts, Fractions, and Pure Compounds. Gorgonian tissues were cut into small pieces and extracted with a mixture of methanol–dichloromethane, 1 : 1 (once), and pure dichloromethane (twice). The extracts were combined and evaporated under reduced pressure. *P. dilatata* crude extract was partitioned between hexane–methanol, yielding a hexane fraction. The methanol layer was evaporated and the residue further partitioned between ethyl acetate and water. Purification of the ethyl acetate fraction by chromatographic methods furnished compounds **1** and **2** (Figure 1). The concentration of **1** and **2** in gorgonian tissue was estimated by thin-layer chromatography and ^{1}H NMR analysis of all semipurified fractions that contain the compounds. Procedures for isolation and structure elucidation of compounds **1** and **2** are described in Martins and Epifanio

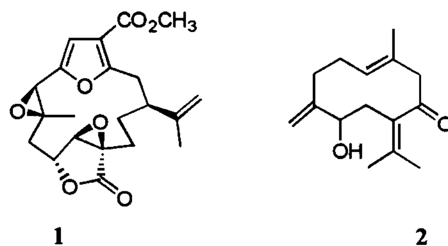

FIG. 1. Terpenoids from *Phyllogorgia dilatata*.

TABLE 1. CONCENTRATIONS OF CHEMICALS IN GORGONIAN FRESH TISSUES[a]

Species	Conc. (mg/cm^3)					
	CE	Hexane	EtOAc	H_2O	**1**	**2**
N. atlantica	35.31					
P. regia	46.12					
P. dilatata	106.58	75.58	3.20	26.16	0.31	0.91

[a]CE = crude extracts; hexane, EtOAc and H_2O = mixtures of compounds with different polarities obtained by partition with these solvents; **1**, **2** = the pure compounds isolated from the ethyl acetate fraction (see Figure 1).

(1998). The concentrations of the chemicals used in the experiments appear in Table 1.

Field Assays with Carrageenan as Solid Matrix. Food strips were prepared by an established methodology (Fenical and Pawlik, 1991; Pawlik and Fenical, 1992) involving a combination of 2.5 g carrageenan (Sigma C-1013 type 1), 60 ml water, and 20 ml commercial tunafish puree packed in oil. Crude gorgonian extracts and *P. dilatata* hexane fraction were incorporated in treated food strips at the same volumetric concentration occurring in the living organisms (Table 1, final volume of 60 cm^3). For each experiment, 20 treated and 20 control strips ($1.0 \times 0.6 \times 5.0$ cm each) were prepared and arranged in pairs and attached to 20 ropes. The ropes were anchored slightly above the bottom at the same sites where the octocorals were collected. Within 3 hr the ropes were retrieved and the amount of each strip eaten was measured. During the experiments several common tropical fishes well known to occur in the studied areas (Nunan, 1992) were observed feeding on test strips (e.g., those belonging to the Families Haemulidae and Labridae at Timbebas reefs; Haemulidae, Labridae, Tetraodontidae, Balistidae, Sparidae and Ostraciidae at Tartaruga beach). Field assays and underwater observations were performed with the aid of a portable surface supply diving apparatus. The Wilcoxon paired-sample test was used to analyze the results [one-tailed (Zar, 1984)].

Field Assays with Calcium Alginate as Solid Matrix. Crude extract, fractions, and pure compounds **1** and **2** in the equivalent amount of 40 cm^3 of *P. dilatata* tissues (Table 1) were reconstituted in a matrix of calcium alginate and assayed at Tartaruga beach. The alginate strips were made by combining 1 g sodium alginate, 34 ml distilled water, and 6 ml commercial tunafish puree (canned tuna in oil, pureed in a minimal volume of distilled water in a blender). The extract, fractions, or pure compounds were added to this mixture without any solvent. The mixture was vigorously stirred and poured into acrylic molds crossed by lengths of cotton string protruding from the ends of the molds. The mixture was hardened by exposure to an aqueous solution of calcium chloride;

exposure was enhanced by punching channels into the hardening mixture with a hypodermic needle. Strips measuring 1.0 × 0.4 × 5.0 cm were sliced with a blade and removed from the molds. Subsequent procedures were identical to those described for the carrageenan strips (Pawlik and Fenical, 1992).

RESULTS

Carrageenan strips that contained *N. atlantica* and *P. regia* crude extracts were consumed more than the controls ($P = 0.0019$ and $P < 0.0001$, respectively, Figures 2 and 3) at Timbebas reefs, Bahia. Compared with controls, both carrageenan and calcium alginate strips containing *P. dilatata* crude extracts sustained less feeding by fishes ($P < 0.0001$) (Figures 2 and 3) at Armação dos Búzios, Rio de Janeiro.

Consumption of carrageenan and calcium alginate strips containing the hexane fraction and calcium alginate strips containing the aqueous fraction of *P. dilatata* did not differ from consumption of control strips ($P = 0.5432$, $P = 0.4507$, and $P = 0.5768$, respectively, Figures 3 and 4). The ethyl acetate fraction deterred feeding on calcium alginate strips ($P = 0.0005$, Figures 3 and 4).

Compounds **1** and **2** (Figure 1) were isolated from the active, ethyl acetate fraction of *P. dilatata* extract. Compound **1**, 11β,12β-epoxypukalide, deterred

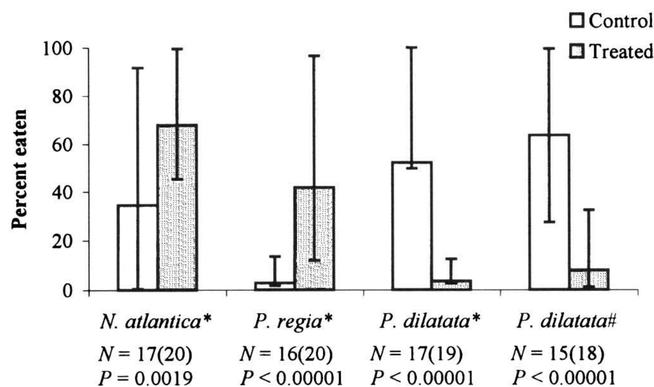

FIG. 2. Consumption by cooccurring fishes of paired bait-strips with (treated) and without (control) crude extracts at the same concentration as found in octocoral tissues. *Carrageenan-based strips. #Calcium alginate-based strips. Error bars are ± SD (data were transformed to SQRT arcsin proportion, SD was calculated and retransformed before plotting). N = no. of paired treated and control strips used in statistical analysis (in parentheses = no. of pairs retrieved of 20 deployed). P = probability calculated from the Wilcoxon paired-sample test, one-tailed.

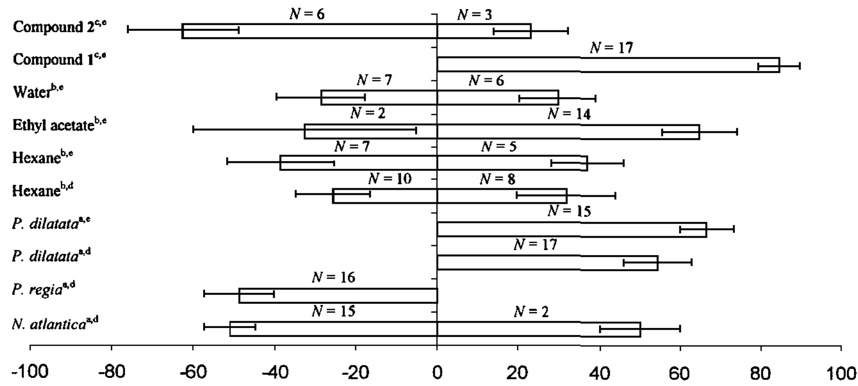

FIG. 3. Mean paired differences in percent eaten between control and treated strips containing crude extracts, fractions, and pure compounds from octocoral. Vertical line through each histogram indicate SE. Superscript a: Crude extracts; b: fractions of *P. dilatata* crude extract; c: pure compounds isolated from the ethyl acetate fraction (see Figure 1); d: carrageenan-based pellets; e: alginate-based pellets; N = number of pairs with negative or positive differences (i.e., control pellets less or more consumed than treatment pellets).

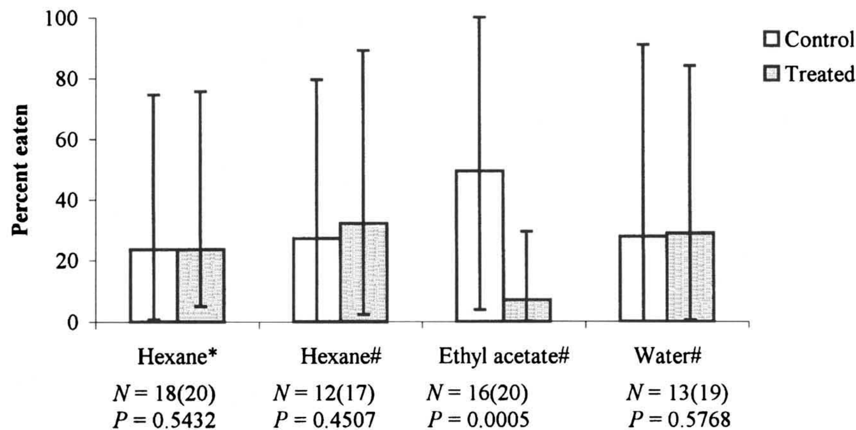

FIG. 4. Consumption by cooccurring fishes of paired bait-strips with (treated) and without (control) fractions of *P. dilatata* crude extracts at the same concentration as found in octocoral tissues. *Carrageenan-based strips; #Calcium alginate-based strips. Error bars are ± SD (data were transformed to SQRT arcsin proportion; SD was calculated and retransformed before plotting). N = no. of paired treated and control strips used in statistical analysis (parentheses indicates no. of pairs retrieved of 20 deployed). P = probability calculated by the Wilcoxon paired-sample test, one-tailed.

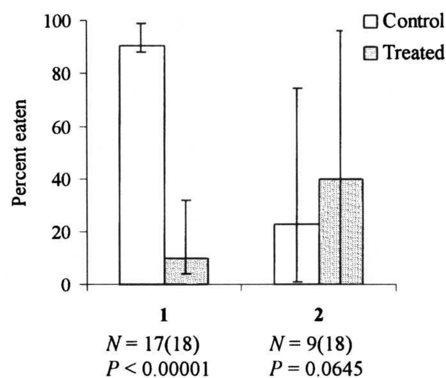

FIG. 5. Consumption by cooccurring fishes of paired calcium alginate-based bait-strips with (treated) and without (control) pure compounds **1** and **2** at the same concentration as found in *P. dilatata* tissues. Error bars are ± SD (data were transformed to SQRT arcsin proportion; SD was calculated and retransformed before plotting). N = no. of paired treated and control strips used in statistical analysis (parentheses indicates no. of pairs retrieved of 20 deployed). P = probability calculated with the Wilcoxon paired-sample test, one-tailed.

consumption by fishes ($P < 0.00001$) (Figures 3 and 5). Compound **2**, isolated for the first time as a natural product (Martins and Epifanio, 1998), did not deter consumption ($P = 0.0645$) (Figures 3 and 5).

Proton NMR analysis of the nonactive hexane fraction of *P. dilatata* showed the presence of a mixture of sesquiterpenes, some of which have been previously isolated from this octocoral (Fernandes and Kelecom, 1995; Martins, 1997; Martins and Epifanio, 1998; Kelecom et al., 1990).

DISCUSSION

Palatability bioassays with food strips or pellets made with polysaccharide matrices have been designed to test the feeding deterrence of marine natural products against fishes (Hay et al., 1998). Fishes comprise the largest group of predators in the sea and presumably play an important role in structuring both temperate and tropical benthic communities. Previous studies of chemical defenses in gorgonians involved different types of Caribbean (Cronin et al., 1995; Fenical and Pawlik, 1991; Harvell et al., 1988; Pawlik et al., 1987; Pawlik and Fenical, 1992), Pacific (Van Alstyne et al., 1994; Wylie and Paul, 1989), and western North Atlantic (Gerhart and Coll, 1993) octocorals, from at least 24 species (Anthothelidae, Briareidae, Gorgoniidae, Plexauridae and Alcyonidae). Although these results revealed a high incidence of crude extracts causing fish

avoidance, to date only seven different natural pure compounds, isolated from six chemically defended octocorals species, have proven to be metabolites responsible for the activity observed (Cronin et al., 1995; Fenical and Pawlik, 1991; Gerhart and Coll, 1993; Harvell et al., 1988; Pawlik and Fenical, 1992; Wylie and Paul, 1989). In the present study we have investigated the occurrence of chemical defenses in three endemic Brazilian octocorals against cooccurring fishes.

Like the Caribbean *Plexaurella* spp. (Plexauridae) studied by Pawlik et al. (1987), Brazilian *P. regia* crude extracts were without apparent predator-deterrence properties; the same was true for *N. atlantica*, the first Nephtheidae (Alcyonacea) octocoral studied. In fact, crude extracts from both these octocorals seemed to stimulate consumption by fishes. These apparently undefended octocorals may use another strategy to avoid predators. One possibility is that their sclerites have an important role in predator deterrence (Harvell et al., 1988; Van Alstyne et al., 1994). We could only isolate hydrocarbonic sesquiterpenes and triglycerides from these extracts (Martins, 1997), which already are known to be inefficient as icthyodeterrent metabolites (Harvell et al., 1988; Fenical and Pawlik, 1991).

In contrast, *P. dilatata* crude extracts, like those from the majority of Caribbean Gorgoniidae (Pawlik et al., 1987), deterred feeding by natural predators. With the goal of isolating the compounds responsible for the observed feeding deterrence, we assayed fractions containing mixtures of compounds with different polarities. For the hexane fraction and crude extract, both carrageenan and alginate strips were employed. These mixtures of metabolites were assayed with two carriers to increase confidence in the results. While the crude extract reduced the consumption of treatment strips, the hexane fraction did not deter feeding by cooccurring fishes when incorporated into either carrageenan or calcium alginate matrices. Among the three fractions tested, only the medium polarity compounds reduced feeding by fishes in the field.

Other studies conducted in our laboratory indicate that the type of polysaccharide matrix used as carriers (baits) can modify the final results when the compounds tested are not stable (Epifanio et al., 1999). These findings led us to add fractions and pure compounds to the alginate matrix, thus avoiding the heating of metabolites that is part of carrageenan strip preparation.

Previous studies of *P. dilatata* revealed that its hexane extract contains one sterol (Kelecom et al., 1980) and two nardosinane sesquiterpenes (Kelecom et al., 1990; Fernandes et al., 1995). Our NMR analysis of the hexane fraction confirmed the presence of such compounds (Martins, 1997; Martins and Epifanio, 1998).

Purification of the ethyl acetate bioactive mixture led to the isolation of two new compounds for this species. Compound **1** is $11\beta,12\beta$-epoxypukalide, a cembranoid diterpene, previously isolated from *Leptogorgia setacea* (Schmitz et al., 1984), and it proved to be the only feeding deterrent metabolite isolated from

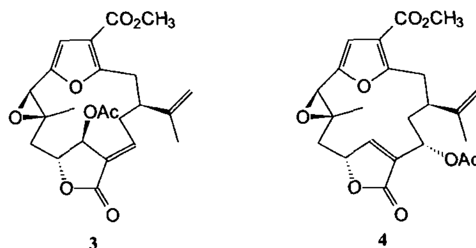

FIG. 6. Icthyodeterrent furanocembranolides from Octocorallia.

the gorgonian tissues. Compound **2** is a new natural germacrane sesquiterpene (Martins and Epifanio, 1998) and was inactive.

Several cembranoid diterpenes have been isolated from octocorals (Faulkner, 1998, and references therein), but only two related furanocembranolides (Figure 6) have been previously investigated as icthyodeterrent compounds by Wylie and Paul (1989) and Gerhart and Coll (1993). The first one, the acetyl derivative **3** isolated from the soft coral *Sinularia maxima*, was offered on squid strips to generalist fishes in Guam. This diterpene deterred feeding at a concentration of 2% dry weight (Wylie and Paul, 1989). The diterpene **4**, isolated from *Leptogorgia virgulata*, induced vomiting in killifish (both animals collected from the North Carolina coast) after incorporation into gelatin pellets at a concentration of 2 mg/cm^3 (Gerhart and Coll, 1993). Both compounds were assayed in higher concentrations than that used for **1** (0.31 mg/cm^3 equal to about 0.41% of pellet dry weight).

In addition to the furanocembranolides of *P. dilatata* and *Leptogorgia setacea* [**1** (Schmitz et al., 1984; Martins and Epifanio, 1998)], *L. virgulata* [**4** (Gerhart and Coll, 1993)] and the soft coral *S. maxima* [**3** (Wylie and Paul, 1989)], others have been isolated from Gorgoniidae octocorals [*Lophogorgia* spp. (Fenical et al., 1981); and *Pseudopterogorgia* spp. (Wright et al., 1989; Chan et al., 1991)]. Apparently, this particular class of compounds has feeding-deterrence properties, protecting these octocorals from different potential fish predators in both tropical and temperate habitats.

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SOCIAL SIGNALS INVOLVED IN SCENT-MARKING BEHAVIOR BY CHEEK-RUBBING IN ALPINE MARMOTS (*Marmota marmota*)

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Abstract—The Alpine marmot *Marmota marmota* is a territorial rodent. Resident adults regularly scent-mark their territory by cheek-rubbing, mainly on burrow entrances and along boundaries. The purpose of this three part study was to gain further insight into this scent-marking behavior by: (1) observing the response of free-ranging marmots to foreign scent marks, (2) confirming the glandular source of the marking substance by histologic examination of the temporal gland, and (3) identifying biologically active chemical fractions of the marking substance. To allow field tests, we developed a device consisting of a glass tube placed upside down over a stake. Two devices were simultaneously placed at one burrow entrance. On one device, a clean tube was used and, on the other, a tube alternatively coated with either whole natural scent-marking substances or various fractions obtained by solvent extraction or chromatographic separation from whole scent-marking substances. Subsequent observations showed a significant difference in the duration of nose contact and number of cheek-rubbing movements. Resident adult marmots sniffed and marked tubes bearing alien marks significantly more than clean control tubes. Similar differences in behavior were observed with ethanol extracts of whole scent-marking substances. Extracts obtained with pentane and dichloromethane showed no bioactivity, suggesting that highly polar compounds are the active substances in the Alpine marmot. The temporal gland is an exocrine gland located on each side of the head

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with numerous pores opening at the surface of the skin in the cheek area. GC-MS analysis of individually collected samples from these glands showed that over 30 compounds were consistently present. Seven of these compounds were identified. Two fractions were obtained and used together and separately in field tests. Fraction 1 was composed mainly of short-chain alcohols and alkanes, and fraction 2 had a more acid and ester composition. The fact that these two fractions were active together but not separately strongly suggests that the active territorial signal results from a synergistic interaction between several compounds.

Key Words—Alpine marmot, *Marmota marmota*, social rodent, chemical communication, scent-marking behavior, scent gland, bioassay, fractionation, mammalian semiochemical.

INTRODUCTION

The Alpine marmot, *Marmota marmota*, is a large rodent (Rodentia: Sciuridae) found exclusively in grasslands above the timberline (Forter, 1975; Herrero et al., 1994). It is a highly social (Barash, 1989), monogamous (but see Goossens et al., 1998), diurnal species living in permanent family units composed of one adult pair and their offspring from successive litters. Reproduction takes place yearly in April immediately after hibernation, and offspring remain in the family unit until at least 2 years of age (Arnold, 1990a,b; Perrin et al., 1993a,b). Family units are territorial. Resident adults carefully scent-mark their territory by regular cheek rubbing behavior and territories are very stable over time (Bel et al., 1995; Coulon et al., 1994; Lenti-Boero, 1995; Perrin, 1993). In a previous paper (Bel et al., 1995), we showed that: (1) peak scent-marking activity occurs between the breeding period and emergence of offspring from the natal burrow, (2) scent-marking mainly involves the main burrow system and boundaries, and (3) foreign scent marks are removed or replaced. The last two findings strongly suggest that scent-marking plays a major role in territorial defense in this species.

The purpose of the present three-part study was to gain further insights into the scent-marking behavior of *Marmota marmota*. We developed a reliable test to determine the response of marmots to foreign scent marks experimentally placed on their main burrow system. The need for such data in free-ranging mammals has been pointed out (Müller-Schwarze et al., 1986), but few assays have been developed (Stralendorff, 1986). Most studies have involved the North American beaver, *Castor canadensis* (Müller-Schwarze, 1992; Müller-Schwarze et al., 1983, 1986; Müller-Schwarze and Houlihan, 1991; Schulte et al., 1994, 1995; Svendsen and Huntsman, 1988). A few marmot species including *Marmota monax* (Meier, 1991), *M. caudata aurea* (Blumstein and Henderson, 1996), and *M. flaviventris* (Brady, 1997) also have been studied in the field.

We also attempted to determine the glandular source of the marking substance. Previous data limit the number of candidate glands. According to Rausch and Bridgens (1989) and Blumstein and Henderson (1996), all 14 species of the genus *Marmota* have two facial glands, i.e., a temporal (or orbital) gland located bilaterally between the eye and the ear and perioral glands located at both oral angles. In the Alpine marmot, cheek glands were reported by Tiedemann (1816) and described in fuller detail by Schaffer (1940). Koenig (1957) linked scent-marking behavior with the deposition of "cheek gland" secretions from adult males. We tested the activity of secretions collected directly from the temporal gland and assessed glandular structure by histological examination.

In the third part of this study, we tried to identify the active chemical components involved in scent-marking behavior of Alpine marmots. Since mammal secretions are complex mixtures, we tested fractions from the marking substance according to a response-guided approach (see Albone, 1984).

METHODS AND MATERIALS

Study Site and Field Observation

This study was conducted at a mean elevation of 2340 m in La Grande Sassière nature reserve (45°29′N, 6°59′E) of Vanoise National Park in the French Alps where we have been studying the marmot population since 1990 (Allainé et al., 1998; Bel et al., 1995; Coulon et al., 1994; Perrin et al., 1993a,b). A total of 427 live-trapped marmots were individually weighed and sexed by measuring anogenital distance (Zelenka, 1965). Based on body mass and head-to-tail length (Zelenka, 1965), animals were classified into the following four age groups: adults 3 years or older, 2-year-olds, yearlings, and juveniles. Animals were marked by tagging and fur dyeing to allow visual identification (Bel et al., 1995; Perrin, 1993). We observed them at a distance of 150–200 m by using 10 × 50 binoculars and a 20–60× telescope (Optolyth TBS 165).

Bioassays

The bioassays described in this report were performed between 1993 and 1997 on eight marmot family units. The test device consisted of two wooden stakes covered by glass tubes (20 cm long × 2 cm diam.) placed near a main burrow entrance at least two days before data recording to give marmots time to become accustomed to their presence. The glass tube used on one stake (treatment tube) had been placed in another family unit two days before so that it bore a natural alien scent mark. The second tube (control tube) was clean. The treatment and control tubes were randomly placed on the right and left of the entrance. The preinstallation time was shorter than in similar experiments on

the golden marmot, *Marmota caudata aurea* (Blumstein and Henderson, 1996), and the woodchuck, *Marmota monax* (Meier, 1991), in which test devices were placed one week prior to observation. Here, we observed that the characteristic, pungent yellowish mucous scent-marks (Bel et al., 1995) were deposited on clean tubes within two days.

As in previous studies on *Castor canadensis* (Butler and Butler, 1979) and *Odocoileus hemionus columbianus* (Müller-Schwarze, 1971; Crump et al., 1984), tests were performed when scent-marking activity is greatest during daylight hours between mid-April and mid-July (Bel et al., 1995). Tests were generally performed in the morning in early spring (about 09:00 hr), and in the morning and afternoon in summer (about 07:30 hr and 17:00 hr). This scheduling strategy was chosen because *M. marmota* shows a progressive seasonal trend to bimodal rhythm (Perrin et al., 1993a,b). As a precaution, no more than one test was performed per day in a given group in order to prevent a possible confounding effect of habituation.

During tests, attention was focused on individuals approaching within 5 m of either test device. After identification of age, sex, and social status, the duration of sniffing (DS), considered as the total duration of the contact between one animal's nose and each tube during one test, was timed by using a stopwatch, and the total number of cheek-rubs (NCR) performed by one animal on each tube during one test was counted. Data were recorded on an audio tape and in notebooks.

Since scent marks deposited by one resident marmot may affect the subsequent response of the other marmots in the family unit, testing was continued as long as none of the marmots in the family group marked either tube. Whenever a resident marmot scent-marked the treatment and/or control tubes, the test was stopped. Tests in which no marmot approached the experimental device or in which no response occurred after 2 hr were stopped and excluded from analysis.

Isolation and Preparation of Active Compounds

Testing Temporal Gland Secretions. Samples were collected from the temporal glands of nine males (seven adults and two 2-year-olds) and 10 females (seven adults and three 2-year-olds). After anesthetization, the temporal region of the head of each animal was shaved. The shaved area was gently massaged to stimulate glandular secretion, and a clean glass tube was rubbed against the skin about three times. One to four tubes were obtained for each animal. Tubes not immediately used were wrapped in clean aluminum foil to protect from sunlight and contamination and stored at −5°C.

Testing Extracts. The compounds deposited on glass tubes were extracted with one of three analytical grade solvents of increasing polarity, i.e., pentane,

dichloromethane, or ethanol. Only one extract with 5 ml solvent was made per tube. After complete evaporation of the solvent, each extract was redeposited on a clean tube and used as the test tube for bioassays. Control test series were also performed to check the response induced by each pure solvent.

Testing Fractions. Only ethanol extracts were submitted to further fractionation after initial bioassays, since they were the only ones showing bioactivity. Chromatography-trapping was carried out on a Girdel 300 gas chromatograph equipped with a packed column (3% CP-Sil5 Chromosorb phase) fitted with an effluent splitter set to a split ratio of 8 : 2. Column temperature was programmed from 60°C to 210°C at a rate of 5°C/min (final time, 10 min; total duration, 40 min). Each ethanolic extract (100 μl) composed of individual temporal gland secretion exudates was coinjected along with an internal standard, *n*-dodecane (n-C_{12}, MW 170), which has a different retention time from any perceptible secretion peak. Chemicals were trapped in a glass capillary tube cooled in Dry Ice. Three volatile fractions were collected. Fraction 1 eluted 10 sec before the standard and fraction 2 was collected from 10 sec after the standard until the end of the program. The total volatile fraction (duration of elution = 40 min) was also collected and bioassayed. Each fraction was subsequently solubilized in 1 ml ethanol (Wheaton vial) and stored at −5°C pending bioassay.

Gas Chromatography–Mass Spectrometry (GC-MS)

Individual ethanol extracts of temporal gland secretions from known males or females (group, age, social status) were used as analytical samples for GC-MS. Temporal secretions were collected in 4-μl glass capillary tubes and stored in 100 μl of pure ethanol. Preliminary analyses were performed on a HPUX-controlled system consisting of a capillary gas chromatograph HP5890 series II coupled to a quadrupole mass spectrometer HP MS Engine 5989A. The chromatograph was equipped with a CPSil 5WCOT apolar column (Chrompack, 25 m, 0.25 mm ID, 0.12 μm DF). Injection was performed in the split–splitless mode (15 sec; injection port 280°C). Oven temperature was programmed from 35°C (4 min isothermal) to 320°C (5 min isothermal) at a rate of 8°C/min. The mass spectrometer was operated in the electron impact mode (GC-MS-EI, 70 eV) and scanning was performed from 40 to 500 uma at the rate of 1 scan/sec.

Data Analysis

Field trials were replicated in various marmot territories. Under the alternative hypothesis, it was assumed that the treatment tubes had been sniffed longer and marked more extensively than control tubes and thus DS and NCR were tested as quantitative variables by using one-tailed nonparametric Wilcoxon matched-pair signed-rank tests (Siegel, 1956).

Study of Glandular Source

Two full-thickness skin samples including the epidermis and the dermis (1 cm^2) were collected from two free-ranging adult marmots shortly after accidental death. One sample was taken from the temporal zone, the zone supposedly containing the glands secreting the compounds involved in scent-marking (Koenig, 1957; Coulon, personal observation). The second sample was collected near the scapula as a control. Samples were fixed with alcoholic Bouin's fixative and stored pending further study. Histological examination was conducted on 7-μm-thick sections cut after dehydration and embedding in paraffin. Sections were stained with Masson's trichome, a variant of Goldner's stain (Martoja and Martoja-Pierson, 1967).

RESULTS

Bioassay

The results of statistical analysis of DS and NCR data obtained in bioassays are shown in Figure 1a and 1b. The treatment tube always elicited a stronger response than the control tube: resident marmots sniffed tubes with natural alien scent marks longer (Wilcoxon test: $N = 15$, $t = 30$, $P = 0.043$, one-tailed) and rubbed them a greater number of times (Wilcoxon test: $N = 13$, $t = 7.5$, $P = 0.0038$, one-tailed) than control tubes. Therefore, while marmots were equally disposed to scent-mark any tube, previously scent-marked or not (see Bel et al., 1995), they responded significantly more to the marked tube than to the clean one.

Temporal Gland Secretions

Thirty-five tests were performed by using extracts obtained from 21 males and 14 females. Temporal gland secretions induced a strong reaction in resident marmots, as for natural scent marks (Wilcoxon tests, male-female secretions, DS: $N = 33$, $t = 39.5$, $P < 0.0001$ one-tailed; NCR: $N = 27$, $t = 28$, $P < 0.0001$ one-tailed) (Figure 1a and 1b).

Secretions from adult males and females were presented separately in order to study responses according to sex. In each test, we measured the difference of both DS and NCR of the treatment and control tube for each marmot. Resulting variables were then used to assess behavioral responses of those animals that had been presented with a male and a female odor in different field tests. The sex of the odor donor did not influence the responses of males or females (Table 1).

Characterization of Chemosignals

Only ethanol extracts induced significant responses. Marmots sniffed tubes marked with an ethanol extract longer (DS Wilcoxon test: $N = 28$, $t = 125.5$, P

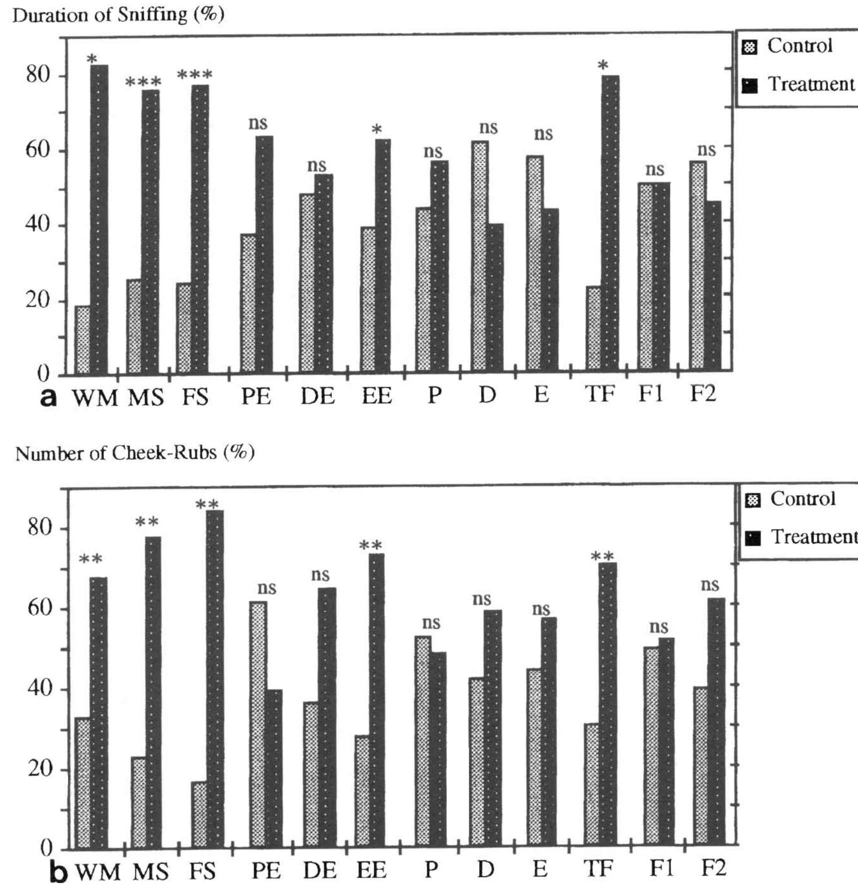

FIG. 1. (a) Relative duration of sniffing (DS) on each tube for different series of tests. Each series of tests is represented by the nature of treatment tube (number of tests): WM (N = 10): whole marks; MS (N = 21): male secretion; FS (N = 14): female secretion; PE (N = 30): pentane extract; DE (N = 13): dichloromethane extract; EE (N = 58): ethanol extract; P (N = 7): pentane; D (N = 10): dichloromethane; E (N = 12): ethanol; TF (N = 18): total fraction; F1 (N = 9): first volatile fraction; F2 (N = 10): second volatile fraction. (b) Relative number of cheek rubs (NCR) on each tube for different series of tests. Each series of tests is represented by the nature of treatment tube; abbreviations as in (a); numbers of tests are given in parentheses. WM (N = 50), MS (N = 21), FS (N = 14), PE (N = 34), DE (N = 21), EE (N = 58), P (N = 7), D (N = 10), E (N = 12), TF (N = 18), F1 (N = 9), F2 (N = 10). Results of Wilcoxon tests are shown (ns: not significant; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; one-tailed).

TABLE 1. PUTATIVE DISCRIMINATION OF TEMPORAL SCENTS ACCORDING TO SEX OF ODOR DONORS AND VARIABILITY AMONG MALE AND FEMALE RESPONSES

	Intensity of marking		Duration of sniffing	
Reactions of females towards male vs female scent-marks[a]	$N = 6$; $t = 5$; $Z = -1.166$		$N = 5$; $t = 4$; $Z = -0.944$	
	$P = 0.2436$	NS	$P = 0.3452$	NS
Reactions of males towards male vs female scent-marks[a]	$N = 5$; $t = 6.5$; $Z = -0.272$		$N = 6$; $t = 8.5$; $Z = -0.420$	
	$P = 0.7855$	NS	$P = 0.6741$	NS

[a]Two-tailed Wilcoxon matched-pairs signed ranks test; here, there are N paired responses of given individuals towards both male and female scents. Z and P values are corrected for ties.

= 0.0385, one-tailed) and marked them more extensively (NCR Wilcoxon test: $N = 11$, $t = 3$, $P = 0.0035$, one-tailed) than control tubes (Figure 1a and 1b). Blank tests with pure solvents ruled out the solvent as the cause of the biological activity of extracts, with results as follows: Wilcoxon tests; DS: pure ethanol: $N = 9$, $t = 13$, $P = 0.1292$, one-tailed; pure dichloromethane: $N = 8$, t 11, $P = 0.1623$, one-tailed; pure pentane: $N = 5$, $t = 5.5$, $P = 0.2904$, one-tailed; NCR: pure ethanol: $N = 4$, $t = 2$, $P = 0.1284$, one-tailed; pure dichloromethane: $N = 5$, $t = 5$, $P = 0.2398$, one-tailed; pure pentane: $N = 6$, $t = 6.5$, $P = 0.1976$, one-tailed).

No significant response was induced by either of the two fractions obtained by fractionation-trapping of the ethanol extract (for DS Wilcoxon tests: Fraction 1: $N = 8$, $t = 17$, $P = 0.4441$, one-tailed; fraction 2: $N = 7$, $t = 8$, $P = 0.153$, one-tailed; for NCR Wilcoxon tests: Fraction 1: $N = 6$, $t = 10$, $P = 0.4578$, one-tailed; fraction 2: $N = 4$, $t = 3$, $P = 0.2286$, one-tailed). However, a significant response was observed from the total fraction (DS: $N = 15$, $t = 28.5$, $P = 0.0366$, one-tailed, NCR: $N = 10$, $t = 2.5$, $P = 0.0051$, one-tailed).

The chemical composition of 24 whole ethanol samples from 18 males and 6 females was analyzed by GC-MS. Major qualitative and quantitative differences were observed. These differences appeared to be unrelated to sex. The 30 most consistently observed peaks, found in more than 50% of individual samples, were selected for further study. A typical total ion chromatogram (TIC) from an adult male is shown in Figure 2. The two fractions used in the bioassay (fraction 1 and fraction 2) showed different GC peaks. Fraction 1 was composed

FIG. 2. (Opposite) Alcoholic extract from temporal secretions of one adule male Alpine marmot: representative total ion chromatography (TIC), analyzed by GC-MS. Preliminary determination could be performed by matching mass spectrum of each peak with those of Wiley and NBS libraries. Fractions 1 and 2, only mixed together once, were biologically active. Fraction 1 is mainly represented by short-chain alcohols (a) and alkanes (h). The second part of the TIC (fraction 2) is characterized by numerous acids (c) and esters (e). Numbers 1–7 refer to precisely identified peaks (see Table 2).

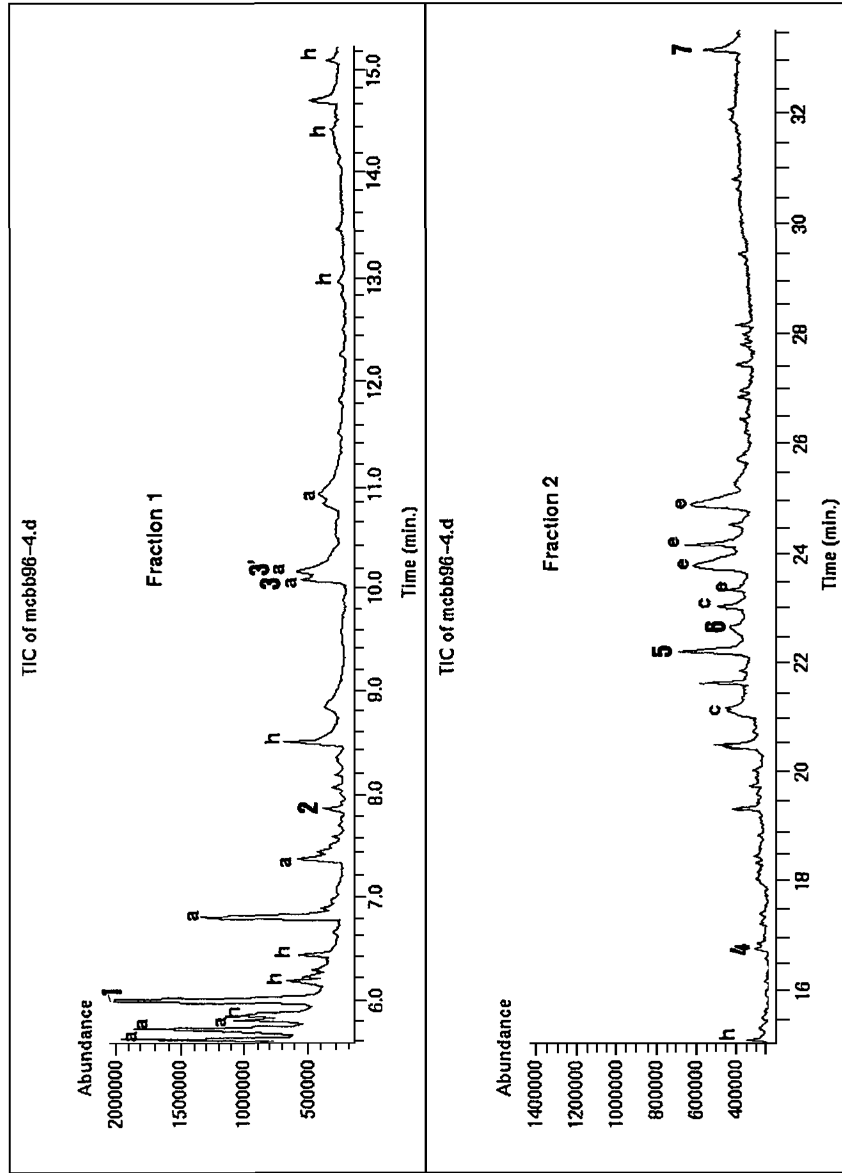
TIC of mcbb96-4.d
Fraction 1
Abundance
Time (min.)
TIC of mcbb96-4.d
Fraction 2
Abundance
Time (min.)

TABLE 2. PRELIMINARY CHEMICAL DETERMINATION OF INDIVIDUAL ETHANOLIC EXTRACTS

Peak	Ret. time (min)	Compound	Formula	Diagnostic ions EI-GC-MS	Reference
1	6	4-Hydroxy-4-methyl-2-pentanone	$C_6H_{12}O_2$	43,59,101 (MW 116)	Hefetz and Lloyd (1993), Finidori-Logli et al. (1996)
2	7.8	Benzenemethanethiol	C_7H_8S	45,65,91,124 (MW 124)	83% Wiley
3	10.08	Nonadien-1-ol	$C_9H_{16}O$	41,55,83,122,140 (MW 140)	70% Wiley
3′	10.16	Nonadien-1-ol (isomer)	$C_9H_{16}O$	41,55,83,122,140 (MW 140)	70% Wiley
4	16.8	2-Ethylamino-2-(2-thienyl)-cyclohexanone	$C_{12}H_{17}NOS$	97,110,123,152,166,195 (MW 223)	87% Wiley
5	22.3	Palmitic Acid	$C_{16}H_{32}O_2$	41,43,57,73,129,185,213,256 (MW 256)	78% Wiley
6	22.6	Musk Ambrette	$C_{12}H_{16}N_2O_5$	77,91,115,146,253,268 (MW 268)	60% Wiley
7	33.2	Cholesterol	$C_{27}H_{46}O$	(MW 386)	95% Wiley

[a]These peaks are also referenced by their number in the total ion chromatogram (Figure 2).

FIG. 3. Temporal gland of an adult Alpine marmot. Schematic distribution of duct openings on the skin surface of a live-trapped adult (the arrow indicates a drop of secretion oozing out of one of the duct openings).

mainly of short-chain alcohols and alkanes, while fraction 2 displayed more acid and ester composition. As listed in Table 2, seven compounds were identified.

The Marking Gland

Macroscopic Findings. Secretory activity seemed to be age-related with no secretion observed in juvenile marmots, while several large drops were obtained from mature animals. However this difference was not tested statistically. The drawing in Figure 3 shows the number and location of pores in sexually mature marmots. More than 30 secretory pores were observed on either side of the face. Pore density was greater in the area between the eye and ear than in the vicinity of the cheek bone. Few, if any, variations of this pattern were observed.

Microscopic Studies. As shown in Figure 4, the temporal gland is an exocrine gland located in the dermis. It is composed of a cluster of specialized interconnected, tubular lobules surrounded by connective tissue and supported by muscle. Secretions drain into a network of interconnecting ducts leading into primary ducts, which end as pores on the surface of the skin. These pores are not associated with hair.

DISCUSSION

Bioassay

As in a previous study (Bel et al., 1995), strong overmarking behavior was observed. Marmots sniffed tubes bearing alien scent marks longer and marked them more extensively than clean tubes. Since these trials were performed with free-living animals, there were no captivity-related confounding effects from

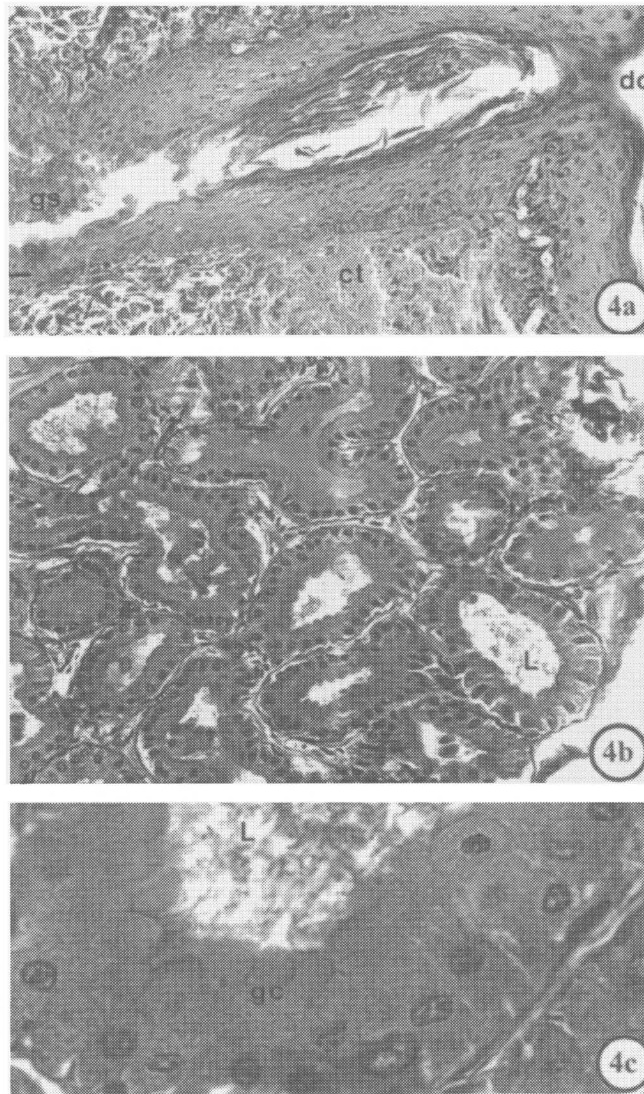

FIG. 4. Temporal scent gland of an adult Alpine marmot. (4a) Transverse section of a glandular duct opening onto the skin surface (do), surrounded by connective tissue (ct) and containing glandular secretion (gs); scale: 100 μm. (4b) Transverse section of a multilobular exocrine skin gland; scale: 100 μm. (4c) Detail of one lobule composed of glandular cells (gc) converging into a central lumen (L); scale: 25 μm.

diet, social system, or territory. Moreover, when meeting foreign marks from conspecifics on their territories, resident marmots also tended to remove or to replace them by their own scents. Thus, this bioassay method provides a simple, reliable method to assess the biological activity of extracts and fractions. In contrast, occurrence of marking was not a discriminant variable (see Bel et al., 1995), probably because marmots are likely to mark any new object found on their territories.

Stralendorff (1986) reported that behavioral responses such as sniffing of chemical signals from conspecifics were rarely specific in mammals and considered olfactory bioassays unsuitable for detection of pheromonelike signals in mammals. However sniffing behavior is often the only reliable means of investigation available for numerous studies of the mammalian olfactory communication system. In our experiments, sniffing behavior together with scent-marking behavior proved to be a powerful tool for assessing the biological activity of a substance. Similarly, in other marmot species, including *M. caligata* (Barash, 1989), *M. caudata aurea* (Blumstein and Henderson, 1996), *M. flaviventris* (Brady, 1997), and *M. monax* (Meier, 1991), sniffing has been used to investigate abilities to discriminate scents from individuals of different sex, familiarity, and/or territorial status.

Although marmots probably sniff odors at a distance, the duration of nose contact with the tubes provides an easy, precise, reproducible criterion. Another advantage is that by comparing duration of nose contact of each marmot towards both tubes, each animal can be used as its own control. In this way an index of the differential reaction of the resident marmot can be determined regardless of the amount of secretion that is deposited. This approach is not sensitive to interindividual or temporary variations in scent-producing ability.

Scent overmarking could serve several purposes in social and territorial species. One possibility is that scent marking is part of a "scent matching" mechanism that provides the basis of territorial maintenance with mutual advantages for the territory owner and intruder (Gosling, 1982). Alternatively, as in the golden hamster *Mesocricetus auratus*) (Johnston et al., 1995) or in the meadow vole *Microtus pennsylvanicus* (Johnston et al., 1997a,b), overmarking could be used by the sexual partner of the group to assess the individual's quality, and counter-marking could strongly influence mate-choice decisions. In the Alpine marmot, overmarking could be used to communicate information to both potential intruders and members of the group, such as the codominant sexual partner.

Marking Gland

Our histological findings support previous data in other marmot species (Rausch and Bridgens, 1989). The results strongly indicate that the temporal or "orbital" glands (Blumstein and Henderson, 1996) are the major source of secre-

tions used for scent-marking in Alpine marmots and suggest that cheek-rubbing is the main method of application. Bioassays determined that secretions from the temporal gland conveyed chemical information between conspecifics. Like golden marmots (Blumstein and Henderson, 1996) and woodchucks (Rausch and Rausch, 1971), adult Alpine marmots exhibit a zone of sparse fur coverage between the eye and ear on both sides of the head. This feature is probably the result of repeated rubbing against rocks, stones and the ground. Microscopic observation confirmed the presence of an apocrine gland in this zone.

Other products have been implicated in scent-marking behavior in mammals. Saliva plays a role in intraspecific communication in domestic cats (Feldman, 1994) and ground-dwelling squirrels (Steiner, 1974). Our results showed that saliva was not necessary to induce significant responses in Alpine marmots. The lack of any perioral secretion (J. L. Clément, personal observation), as well as of any particular smell in the perioral area, suggests that no functional perioral gland exists in this species. Feces and urine left in the territory also may convey chemical information between conspecifics, but we did not study those behaviors. Our results (Bel et al., 1995; this study) suggest cheek-rubbing to be the main marking mode.

Fractionation

This is the first time that fractionation has been used to study chemical communication in a marmot species. For this purpose we devised special techniques to allow field collection and analysis of pure and concentrated material. To minimize the effects of aging of scent-marks, which has been reported in some mammals (Clark, 1982; Johnston et al., 1995), all field trials were performed by using either fresh samples or samples that had been frozen immediately after collection.

Pentane, dichloromethane, and ethanol were used for extraction. Some compounds in the three extracts might be the same but only the ethanol extract was biologically active in eliciting a response similar to that of natural secretions. Pentane extracts showed no biological activity, and dichloromethane extracts induced only a slight, nonsignificant response. These findings suggest that the most polar fraction contains most of the active compounds. Although not commonly used for GC analysis, ethanol allowed successful solubilization of active compounds in the North American beaver (Svendsen and Huntsman, 1988; Schulte et al., 1995).

Neither of the two subfractions (fractions 1 and 2) exhibited bioactivity. There are two possible explanations for this result. The first may be that separation resulted in a selective loss of material. Molecules interacting specifically with active compounds could have been altered or too heavy to elute (e.g., proteins). The second explanation may be that detection of chemosignals by Alpine

marmots requires a threshold concentration. This requirement has been noted in many other species. Our results suggest that the active signal may be composed of a mixture of different molecules interacting synergistically (see Müller-Schwarze et al., 1986). Fractionation probably separated molecules that must interact to form a functional complex.

Most studies have concluded that chemosignals in mammals are highly complex and many compounds that interact to form functional mixtures have been identified (Stralendorff, 1987; Houlihan, 1989; Jemiolo et al., 1989; Müller-Schwarze and Houlihan, 1991; Schulte et al., 1994). Findings have generally been in agreement with the "social odor hypothesis," which states that mixtures are more active than single compounds and larger mixtures more active than smaller ones.

Albone (1984) stated that the "response-guided" strategy "is of only limited applicability in mammals," because chemosignals are complex mixtures. However, response-guided studies on semiochemicals have been successful in the North American beaver (Müller-Schwarze and Houlihan, 1991; Müller-Schwarze, 1992; Schulte et al., 1994, 1995), mice (Jemiolo et al., 1992, 1994; Singer et al., 1993), tree shrews (Stralendorff, 1987), and black-tailed deer (Müller-Schwarze, 1971; Crump et al., 1984). Further experiments with the fractionation-bioassay procedure are needed to identify the compounds underlying chemical communication in Alpine marmots.

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POLYMORPHISM IN PLANT DEFENSE AGAINST HERBIVORY: CONSTITUTIVE AND INDUCED RESISTANCE IN *Cucumis sativus*

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Abstract—Theory predicts that plant resistance to herbivores is determined by both genetic and environmentally induced components. In this study, we demonstrate that the phenotypic expression of plant resistance to spider mite herbivory in *Cucumis sativus* is determined by genetic and environmental factors and that there is an interaction between these factors. Previous feeding by spider mites induced systemic resistance to subsequent attack over several spatial scales within plants, reducing the population growth of mites compared to that on control plants. Effects of induction were effective locally over the short term, but resulted in local increased susceptibility to spider mite attack after several days. However, this local induced susceptibility on the damaged leaf was associated with induced resistance on newer leaves. Induced resistance was correlated with increases in cucurbitacin content of leaves, but was not associated with changes in the density of leaf trichomes. Induced resistance to herbivory was not detected in plants of a genotype lacking constitutive expression of cucurbitacins, which were in general highly susceptibile to mite attack. Allocation trade-offs between growth and defense are often invoked to explain the maintenance of variation in the levels of plant resistance. Contrary to current thinking, neither constitutive nor herbivore-induced plant resistance were associated with reductions in plant allocation to root and shoot growth. However, plants that had high levels of induced resistance to spider mites were the most susceptible to attack by a

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specialist beetle. Such ecological trade-offs between resistance to generalist herbivores and susceptibility to specialist herbivores may be important in the maintenance of variation of plant resistance traits. In summary, *C. sativus* exhibits strong genetic variation for constitutive and induced resistance to spider mites, and this variation in resistance is associated with ecological trade-offs.

Key Words: Cucumber, *Cucumis*, cucurbitacins, defense theory, *Diabrotica*, herbivory, induced responses, plant-insect interactions, plant resistance, spider mites, *Tetranychus*.

INTRODUCTION

Host-plant genotype and environment can play central roles in determining plant reistance to herbivory (Denno and McClure, 1983; Fritz and Simms, 1992). Intraspecific genetic variation can cause large differences in plant resistance to herbivores (Berenbaum et al., 1986; Simms and Rausher, 1989; Karban, 1992). Environmentally induced effects, such as plant responses to herbivory, have also been shown to negatively impact preference and/or performance of herbivores in over 100 systems (Karban and Baldwin, 1997; Agrawal, 1998a; Agrawal et al., 1999a). In addition, plant genotype and environment frequently interact to influence plant resistance to herbivores (Agrawal, 1999a). Determining the factors that maintain variation in constitutive and induced levels of plant resistance has been a central issue in plant defense theory (Herms and Mattson, 1992). Experimentally determining costs and benefits of expressing particular resistance phenotypes will help us to understand the selection pressures and constraints that have evolutionarily shaped plant defense.

Current thinking suggests that constitutive and environmentally induced expression of plant resistance traits diverts resources otherwise available for growth and reproduction (Herms and Mattson, 1992). Such trade-offs have been commonly invoked and modeled as mechanisms behind the maintenance of genetic variation in plant resistance and inducible or facultative expression of plant resistance (environmentally induced variation). In a critique of this "allocation model," Mole (1994) pointed out that allocation to plant resistance may be trivial, especially in light of the myriad functions that plants perform. Plants do not simply grow or defend. In addition, there is increasing evidence that plants can increase nutrient uptake and photosynthetic rate to compensate for particular energetic burdens such as herbivory (van der Meijden et al., 1988; Trumble et al., 1993; Rosenthal and Kotanen, 1994; Sadras, 1995; Strauss and Agrawal, 1999).

Some authors have recently suggested non-mutually exclusive alternative hypotheses to the allocation model for explaining variation in plant resistance traits (Linhart, 1991; Parker, 1992; Simms, 1992; Karban, 1993; Rausher, 1996; van der Meijden, 1996; Agrawal and Karban, 1999). However, experimental tests

of these non-mutually exclusive hypotheses are lacking. One hypothesis suggests that variation in plant resistance traits should be maintained in both constitutive and induced resistance because some specialized herbivores may have counter-adapted to the defense (Linhart, 1991; Adler and Karban, 1994; van der Meijden, 1996; Agrawal and Karban, 1999). For example, if specialized herbivores make use of plant defense effective against generalist herbivores as host finding cues or as a source of chemicals that they can sequester for their own defense, then the "plant resistance traits" may only be beneficial to the plant in some environments (i.e., where generalist herbivores are more common than specialized herbivores). Anecdotal observations of such trade-offs between resistance and susceptibility to generalist and specialist herbivores have been reported from several plant–herbivore systems (Chambliss and Jones, 1966; Da Costa and Jones, 1971; Matsuda, 1988; Wink, 1988; Linhart, 1991; Pasteels and Rowell-Rahier, 1992; Vaughn and Hoy, 1993; Landau et al., 1994; Matsuki and MacLean, 1994; Giamoustaris and Mithen, 1995; Loughrin et al., 1995, 1996; Mithen et al., 1995; Alados et al., 1996; Jiang et al., 1997 van Dam and Hare, 1998).

In this study, we document differences in plant resistance to herbivory in two near-isogenic varieties of *Cucumis sativus* that differ qualitatively in production of cucurbitacins (hereafter, high cucurbitacins = bitter plants and no cucurbitacins = sweet plants). We first demonstrate differences in constitutive and inducible levels of plant resistance to generalist spider mites (*Tetranychus urticae*) in bitter and sweet plants and then investigate the phytochemical and physical basis of this resistance. We test two models, the allocation trade-off model and the specialist/generalist trade-off model, to explain the genotype and environmentally induced variation in resistance. Specifically, we asked: (1) What are the consequences for spider mite populations feeding on bitter or sweet, previously damaged or undamaged *C. sativus* plants? (2) Do cucurbitacins or trichomes increase locally and systemically in damaged plants? (3) What are the within plant spatial aspects of induced resistance in *C. sativus?* (4) Is constitutive or induced resistance to spider mites correlated with reduced allocation to root and/or shoot growth and biomass accumulation? (5) Is there a trade-off between resistance to generalist herbivores and susceptibility to specialist herbivores such that specialist herbivores (spotted cucumber beetles, *Diabrotica undecimpunctata*) prefer to feed on plants expressing high levels of induced resistance to spider mites?

Study System. *Cucumis sativus* (Cucurbitaceae) is commonly grown as cultivated cucumber. Many cucurbits vary qualitatively in the intraspecific presence or absence of cucurbitacin production (tetracyclic triterpenoids). Cucurbitacins are the most bitter tasting compounds known and can be detected by humans in dilutions of 1 ppb (Da Costa and Jones, 1971; Metcalf et al., 1980; Metcalf and Lampman, 1989). We used Marketmore varieties 76 (bitter) and 80 (sweet), that are genetically identical except for those genes tightly linked

with the gene responsible for the presence or absence of cucurbitacins (H. M. Munger, personal communication). Cucurbitacin production appears to be controlled by a two-allele single-locus gene, where cucurbitacin production is dominant. Cucurbitacins have been demonstrated to function defensively against several widespread generalist herbivores including spider mites, roaches, several beetles, lepidopteran larvae, mice, and vertebrate grazers (Da Costa and Jones, 1971; Gould, 1978; Metcalf and Lampman, 1989; Tallamy et al., 1997). Specialized feeders on *C. sativus*, such as diabroticite beetles, however, use cucurbitacins as kairomones, which cause "compulsive" feeding on plants with high levels of cucurbitacins (Chambliss and Jones, 1966; Metcalf et al., 1980, 1982; Metcalf and Lampman, 1989). In addition, diabroticite beetles sequester cucurbitacins, which may protect them against attack by natural enemies (Ferguson and Metcalf, 1985; Barbercheck et al., 1995). Growth rate, fecundity, and longevity of these beetles is unaffected by cucurbitacins (Hirsh and Barbercheck, 1996; Tallamy and Gorski, 1997).

METHODS AND MATERIALS

Cucumis sativus plants were grown in 125-cm^3 plastic pots in greenhouse soil (U.C. mix, Redi Gro, Inc., Sacramento, California) in laboratory growth boxes at ambient temperatures (25 ± 3°C) on a 14L:10D cycle. Two-spotted spider mites were collected from cotton, beans, roses, and morning glories in Davis, California, and were maintained in a laboratory colony on cotton plants.

Genetic Variation in Constitutive and Induced Resistance to Spider Mites. This experiment was set up as a 2 × 2 factorial design to measure spider mite population growth on bitter and sweet plants and on plants with and without initial plant exposure to spider mites. The protocol for the initial spider mite exposure and the challenge with subsequent mites was as follows: When the cotyledons had fully expanded (after seven days), half the plants were inoculated with 15 adult female mites. They were allowed to feed on the cotyledons for three days, after which all of the plants (including undamaged controls) were dipped in dicofol (Kelthane, 100 ppm, Rohm and Haas, Philadelphia, Pennsylvania), a miticide that does not move systematically through the plant. At this time, the first true leaf was only a bud. Seven to 10 days later, when the first true leaf had fully expanded, this leaf was isolated by placing a thin ring of sticky Tanglefoot (Grand Rapids, Michigan) around the petiole near the base. Each first true leaf was inoculated with three adult female spider mites. Seven days later all stages of mites were counted on the leaves with the help of a dissecting microscope. This experiment was conducted three times with sample sizes of 10–20 plants for each treatment in each trial. Spider mite population growth on bitter and sweet, and induced and not induced plants was analyzed for the three trials by using a three-way ANOVA.

Cucurbitacin and Trichome Analysis. Sweet *C. sativus* plants did not contain cucurbitacins (H. M. Munger, personal communication; A. A. Agrawal, personal observation by taste). To evaluate local and systemic induction of cucurbitacins in bitter plants, experiments identical to those described above (without the challenge) were carried out with 12 plants in each of the two treatments. When the first true leaves had fully expanded, cotyledons and first true leaves were severed by using a razor blade, placed in coin envelopes, and put in a 0°C freezer. The following day, leaf tissue was freeze-dried and weighed. Because first true leaf samples were too small for chemical analysis, the 12 samples were randomly divided into four groups of three leaves each. Leaf samples were dried at 50°C over night and milled to a fine powder. We extracted 0.2 g of leaf powder in 0.5 ml of 95% methanol in 1.5 ml Eppendorf tubes. Homogenates were vortexed three times and allowed to sit overnight. The next day, homogenates were vortexed again, centrifuged, and the pellets were extracted two more times at 1-hr intervals with methanol. Combined methanol extracts were dried under N_2. Samples were redissolved in methanol and spotted on silica gel 254 thin-layer chromatography NP plates along with cucurbitacin C standards. Plates were developed in ethylacetate–toluene (75 : 25), dried, and cucurbitacin aglycones were quantified by using a Shimadzu SC9000U scanner (Shimadzu Scientific Instruments, Columbia, Maryland) (Halaweish and Tallamy, 1993). Cucurbitacin C was identified by comparing R_f values with those of the standard (visualization: UV). Cucurbitacin content was compared between induced and noninduced plant tissue by using one-tailed t tests to address the a priori hypothesis that cucurbitacin content would be higher in more resistant (induced) leaves.

An additional set of chemical analyses was conducted to test for phytochemical effects of localized damage to the first true leaf of bitter plants. We grew 40 plants until the first true leaf was fully expanded and then inoculated 20 of them with 15 spider mites each. The mites were allowed to feed for three days, after which all plants were treated with the miticide (dicofol). These plants were not challenged with mites, but were allowed to grow for three additional days, after which the damaged leaves were harvested, as above, for cucurbitacin analysis. Again, several leaves were pooled for each sample and the final sample sizes were: control, $N = 6$; induced $N = 8$.

In addition to the cucurbitacin analyses, we counted trichomes on the undersides of induced and control leaves to determine if part of the induced resistance to spider mites seen in the bitter plants were correlated with increased density of trichomes ($N = 15$–17 for each treatment). The total number of trichomes was counted in a 1-cm square at the base of each leaf, and the means were compared by t test.

Within-Plant Distribution of Induced Resistance. We conducted several experiments to determine spatial aspects of the induced resistance of *C. sativus* to spider mite herbivory. The protocol used in these experiments was identical to

that described above except as indicated. In the first experiment, we challenged plants by placing 30–50 mites on the first true leaf (N = 11 plants for each treatment). At this time, the second true leaf was beginning to expand, and neither leaf was isolated with Tanglefoot. The total number of mites was counted on the first and second true leaves one week later. The goal was to determine if spider mite herbivory induced systemic resistance throughout the entire growing plant and to test if induced resistance was robust to a challenge of mites mimicking outbreak conditions. In the second experiment, we induced the first true leaf (instead of the cotyledons) when it was fully expanded, inoculated the second true leaf with three adult female mites, and determined population growth after a week (N = 10–12 for each treatment).

In the third set of experiments, we tested for localized effects of induction by creating two 3-cm^2 Tanglefoot enclosures on the tops of all first true leaves (see Gould, 1978). The two square enclosures were contiguous and shared one common border. On treatment plants, we inoculated one enclosure with 15 mites and then removed them by using the miticide (dicofol) after three days (one enclosure on the control leaves was also treated with the miticide). After three days, the second enclosure on all plants was inoculated with three female mites, and mites were counted after one week. In this experiment, the challenge mites were exposed to a section of the leaf unexposed to initial treatment with mites. This experiment was repeated two times with 11–13 samples for each treatment, each trial. Because the results were contrary to our predictions, we conducted an additional experiment on localized effects of herbivory on subsequent plant resistance. We inoculated the first true leaf with 15 spider mites, and after three days of feeding we removed all mites and eggs by hand by using a paint brush and a dissecting microscope (no Tanglefoot or miticide was used on the leaf). After three days, we inoculated the same leaf and controls with three mites and measured mite population growth as above (N = 19–20 for each treatment). In this experiment, the challenge mites were exposed to the entire first true leaf (as were the initial treatment mites). The goal of this experiment was to test for localized effects of mite feeding on subsequent mite population growth without the use of Tanglefoot or a miticide.

A final experiment on localized effects of damage was conducted to test if induced responses conferred rapid resistance to herbivory. In the above experiments, challenge mites were placed on the plant six days after the original inducing mites were placed on the plant; in this experiment, challenge mites were placed on the plant after one day of mite feeding. The experiment was conducted by using the Tanglefoot enclosures and miticide procedures described above. Treatment plants (N = 18) were exposed to feeding by 15 mites for the first 24 hr, but control plants (N = 16) received no mites for the first 24 hr. After the mites were removed by hand, all plants were inoculated with three female mites, and mites were counted after one week. Mite population sizes in these experiments were compared by using t tests.

Growth Rate of Bitter and Sweet Plants. To test if plant resistance and associated cucurbitacin production were correlated with growth rate and/or allocation to root and shoot biomass, we set up a 2 × 2 factorial experiment that used bitter and sweet plants initially exposed or not exposed to spider mites (N = 24–25 for each treatment). As before, plants in the induction treatment were exposed to mites for three days. All plants were treated with the miticide, but no plants were subsequently challenged with spider mites. Plants were grown for two weeks following initial spider mite exposure and then were harvested. Roots were washed with water to remove all soil and all plant parts were dried for three days at 60°C in a drying oven. Dried plant material was weighed to 0.1 mg, and differences in weight were analyzed by using two-way ANOVAs.

Feeding Preference of a Specialized Feeder. Spotted cucumber beetle (*Diabrotica undecimpunctata*) adults were collected from cotton flowers in Davis, California, and used immediately for experiments. Individual *C. sativus* plants that were induced or not induced were prepared as above by damaging cotyledons and then plants were paired in 20-liter ventilated boxes. Two days after damage, one beetle was introduced to each cage and allowed to feed for 24 hr; the percent leaf area consumed was estimated on each plant by using an acetate grid (N = 10 pairs). Measurements of percent leaf area consumed were arc-sine square root transformed and compared by paired t test.

RESULTS

Genetic Variation in Constitutive and Induced Resistance to Spider Mites. Spider mites had approximately twice the population growth on sweet plants lacking cucurbitacins than on bitter plants with cucurbitacins (Figure 1, Table

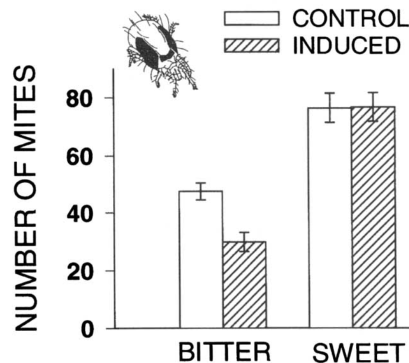

FIG. 1. The influence of plant genotype and induction treatment on population growth of herbivorous spider mites (mean ± SE).

TABLE 1. ANALYSIS OF VARIANCE TABLE FOR EFFECTS OF PLANT GENOTYPE AND INDUCTION TREATMENT ON SPIDER MITE POPULATION GROWTH

Source	*df*	ms	*F*	*P*
Genotype	1	37534.818	106.786	<0.001
Induction	1	2542.079	7.232	0.008
Genotype × induction	1	2361.010	6.717	0.011
Trial	2	4463.464	12.698	<0.001
Error	138	351.497		

1). Although previous mite damage (induction) had no effect on subsequent plant resistance in sweet plants, bitter plants showed a 40% increase in resistance following an initial mite attack (Figure 1, Table 1). Overall, there were highly significant effects of genotype (sweet vs. bitter), induction, and genotype × induction interaction on population size of the mites, indicating that although damage induced resistance in bitter plants, sweet plants were not inducible (Figure 1, Table 1).

Cucurbitacin and Trichome Analysis. Induced resistance caused by initial mite attack was associated with a significant increase in the cucurbitacin content of leaves of bitter plants (Figure 2). The cucurbitacin content of induced bitter plants increased locally in the (damaged) cotyledons by 30% (t = 2.328, df = 20, P = 0.015) and systemically in the (undamaged) first true leaves by 50% (t = 2.368, df = 6, P = 0.028) over controls. The number of trichomes on induced and control plants did not differ (mean ± SEM of control plants = 31.643 ± 1.856; induced plants = 30.353 ± 1.025; t = 0.637, df = 29, P = 0.529).

Within Plant Distribution of Induced Resistance. Induced resistance to spider mites was spatially dynamic within individual plants: when previously dam-

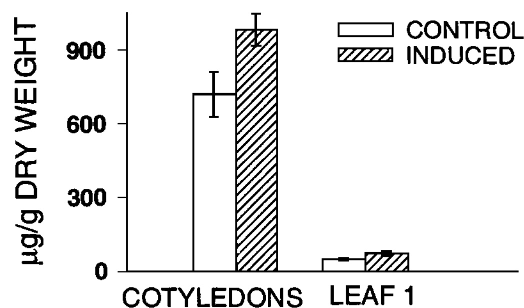

FIG. 2. Local and systemic effects of spider mite herbivory on cucurbitacin-c content of leaves (mean ± SE).

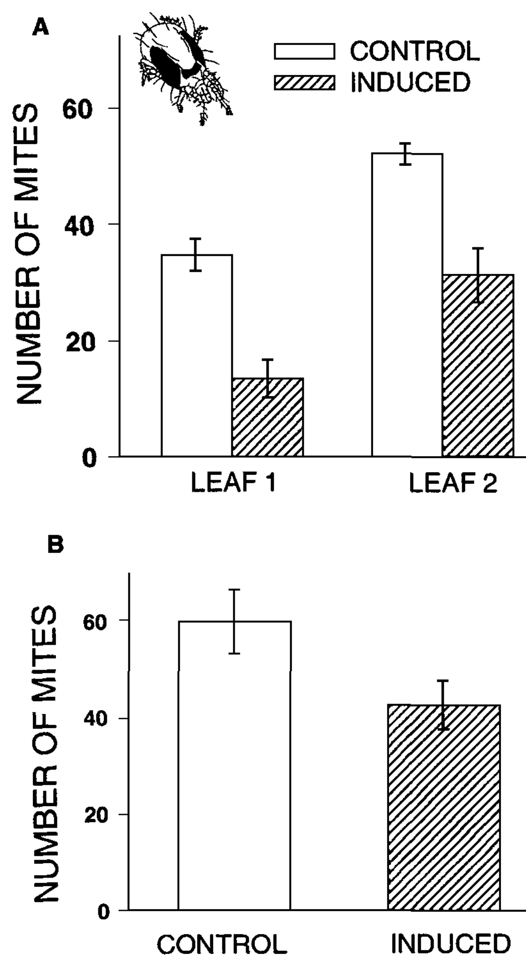

FIG. 3. Within-plant spatial aspects of induced resistance to spider mite herbivory (mean ± SE): (A) Effects of initial spider mite damage to the cotyledons on systemic resistance on the first and second true leaves under outbreak conditions. At the time of initial spider mite attack, the first and second true leaves were not formed. (B) Effect of initial spider mite damage to the first true leaf on spider mite population growth on the second true leaf.

aged plants were faced with a challenge of 30–50 spider mites, induced resistance persisted and decreased mite population growth twofold compared to controls (Figure 3A) ($F_{1,40}$ = 39.358, P < 0.001). The induced resistance was systemic throughout the entire plant; only the cotyledons were damaged, and mite

population growth was decreased on both the first and second true leaves (Figure 3A). The second true leaf consistently supported higher spider mite populations than did the first true leaf. When only the first true leaf was damaged, the quality of second declined and supported smaller mite populations compared to undamaged controls (Figure 3B, $t = 2.134$, $df = 19$, $P = 0.046$).

Damage to the first true leaf resulted in increased susceptibility of the first true leaf to subsequent attack in the experiments that used Tanglefood enclosures (Figure 4A; induction effect: $F_{1,44} = 6.234$, $P = 0.016$; trial effect: $F_{1,44} = 0.036$, $P = 0.851$). These efects were found in plants that were challenged locally with mites six days after the initial attack. Similar results were found when we locally challenged leaves with mites after removing the initial mites by hand (Figure 4B, $t = -7.422$, $df = 37$, $P < 0.001$), not using Tanglefoot or the miticide. Leaf cucurbitacin analysis on plants treated exactly as in the above two trials revealed that there was two times the level of cucurbitacins in locally damaged leaves compared to undamaged leaves (mean ± SEM μg/g dry weight cucurbitacin, controls = 5.5 ± 1.016, induced = 10.175 ± 1.406; $t = -2.695$, $df = 12$, $P = 0.020$). This result was contrary to our predictions because increased susceptibility to mites was correlated with increases in cucurbitacin content of leaves. However, when leaves that were damaged by spider mites were challenged 24 hr after the initial inoculation, we found increased resistance on plants that were previously damaged (Figure 4C, $t = 2.506$, $df = 32$, $P = 0.018$). These results suggest that local induced responses can result in short-term increased resistance followed by a rapid decay and increased susceptibility.

Growth Rate of Bitter and Sweet Plants. Neither root nor shoot biomass accumulation was affected by constitutive or induced levels of plant resistance (Figure 5, all $Fs_{1,40} < 1.13$, all $Ps > 0.291$).

Feeding Preference of a Specialized Feeder. Diabrotica undecimpunctata beetles ate significantly more leaf tissue of previously damaged plants compared to undamaged controls (Figure 6, $t = -3.695$, $df = 9$, $P = 0.005$).

DISCUSSION

Genetically determined expression of constitutive and induced plant resistance traits can have important fitness consequences for herbivores and the plants they feed on (Agrawal, 1999a). In the present experiment, spider mites produced nearly two times as many progeny on plants that did not produce cucurbitacins compared to near-isogenic plants that constitutively produced cucurbitacins. In addition, systemically induced production of cucurbitacins was correlated with reductions in mite population growth on previously damaged plants compared to controls. Sweet plants did not exhibit induced resistance to herbivory by spider mites. This finding has two important implications: First, the induced resistance

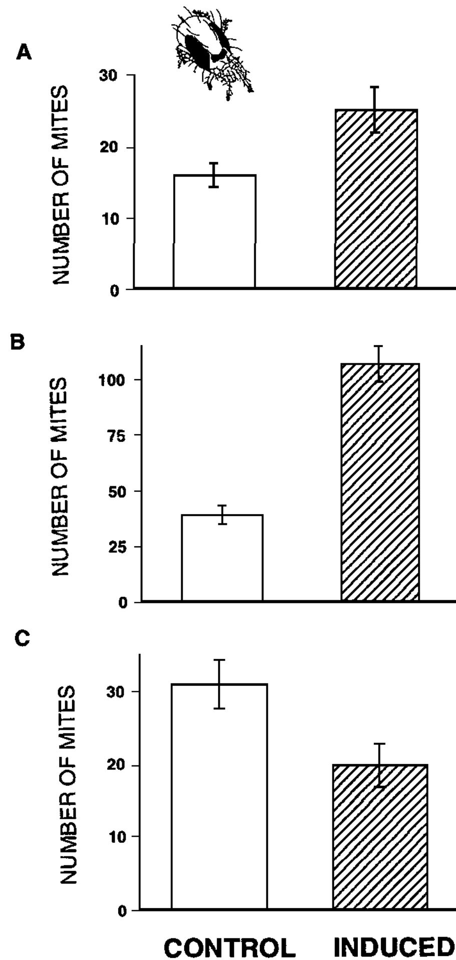

FIG. 4. Localized effects of induction (mean ± SE): (A) Effect of initial spider mite damage to the first true leaf on subsequent mite population growth on the same leaf, six days after the original inoculation. Initial mites and challenge mites were confined by using Tanglefoot enclosures. Initial mites were removed with a miticide. (B) Effect of initial spider mite damage to the first true leaf on population growth of mites subsequently attacking the same leaf, six days after the original inoculation. Initial mites and challenge mites were free to move across the entire first true leaf. All mites and eggs were removed by hand after initial attack. (C) Effect of initial spider mite damage to the first true leaf on subsequent mite population growth on the same leaf, one day after the original inoculation. Initial mites and challenge mites were confined by using Tanglefoot enclosures. Initial mites were removed with a miticide.

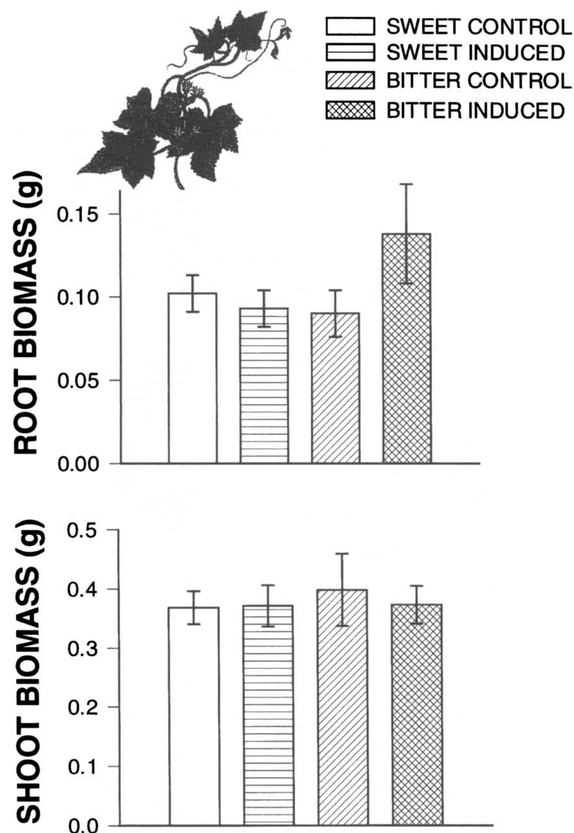

FIG. 5. The influence of plant genotype and induction treatment on allocation to root and shoot biomass (mean ± SE).

demonstrated in bitter plants was likely due to phytochemical induction of cucurbitacins and probably not due to nutritional effects, the induction of unidentified compounds, or deterioration of host-plant quality. Elsewhere we have demonstrated that when cucurbitacins are added to a benign host plant, mite population growth is reduced compared to that on controls (Agrawal, 1999b). Second, the single locus that appears to be responsible for constitutive production of cucurbitacins in *C. sativus* is also associated with inducible expression of cucurbitacins. Plants with constitutive expression of cucurbitacins are inducible, and plants without constitutive cucurbitacins are not inducible. This result violates theoretical predictions of a negative association between constitutive and inducible resistance in plants (Herms and Mattson, 1992). Zangerl and Beren-

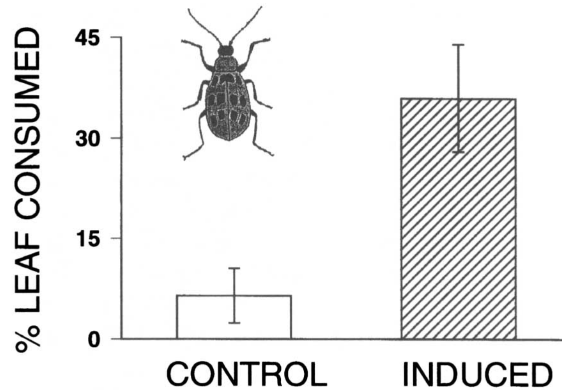

FIG. 6. The effect of induction treatment in the bitter genotype on feeding of a specialist Diabroticite beetle (mean ± SE).

baum (1990) and Siemens and Mitchell-Olds (1998) also found a positive relationship between constitutive and inducible resistance in wild parsnip and mustard, respectively. Other workers have found no apparent association between constitutive and induced plant resistance to herbivores (Brody and Karban, 1992; Thaler and Karban, 1997).

Induced resistance to spider mites in *C. sativus* is spatially dynamic within individual plants (Figure 3). Resistance was induced systemically throughout the plant when cotyledons were damaged. Damage to the cotyledons led to more resistant first true leaves compared to undamaged controls, even though the first true leaf was not fully formed when the damage took place. Similarly, we found systemic effects of damaging the first true leaf, which resulted in a more resistant second true leaf compared to undamaged controls. Finally, induced resistance to spider mites is a robust plant response that did not collapse or lose its effectiveness when faced with outbreak conditions where up to 50 mites infested the leaves. In other systems, such as the pine–bark beetle association, large numbers of attackers have been shown to overwhelm the induced host-plant defenses (reviewed by Tallamy, 1986).

Localized responses to leaf damage appeared to be more complex than systemic responses. In two types of experiments set up to detect localized induced resistance, initial damage actually increased susceptibility to subsequent attack (after short-term induced resistance), greatly increasing mite population growth on the previously damaged leaf (Figure 4). This susceptibility was found on plants that were initially damaged six days earlier. In addition, cucurbitacin content of damaged leaves was two times higher than in control leaves. This result is contrary to our predictions of cucurbitacins increasing resistance to the spi-

der mites. On plants that were damaged in the past 24 hr, we did find induced resistance locally. These results suggest that local induced responses can result in increased resistance or susceptibility, depending on the timing of subsequent attack. Similar results were recently reported by Underwood (1998), in which damaged soybean plants initially became more resistant to beetles; however, this resistance decayed and eventually led to induced susceptibility. Although some cucurbit responses to herbivory yield rapid increases in cucurbitacins followed by subsequent relaxation of cucurbitacins (Tallamy and McCloud, 1991), our phytochemical analysis suggests that cucurbitacins were higher in locally damaged leaves, even those that were more susceptible to herbivores. Thus, even though there was increased local allocation to defense, the localized herbivory may have caused the damaged leaf to become a nutrient sink (e.g., Inbar et al., 1995), increasing the nutritive value of the leaf to the herbivores, and leaving the leaf more susceptible to herbivores several days after attack.

In addition to the direct effects of induced plant responses to herbivory, damage to some plants results in the release of volatiles that attract natural enemies of herbivores (Takabayashi and Dicke, 1996; Shimoda et al., 1997; Agrawal, 1998b; De Moraes et al., 1998; Thaler, 1999). Young *C. sativus* plants that were infested with spider mites released a blend of volatiles that attracted the predaceous mite, *Phytoseiulus persimilis* (Takabayashi et al., 1994). Taken together, our study and that of Takabayashi et al. indicate that when *C. sativus* is damaged by spider mites, the plants not only increase defense directly by increasing compounds that reduce herbivore population growth, but also indirectly by attracting natural enemies. At this stage, it is not clear whether sweet plants (lacking cucurbitacins and induced resistance) would also produce the blend of volatiles that attract natural enemies of herbivores. Recent evidence from systems involving beet army worms, corn, and tomato plants indicated that induced emission of natural enemy attractants is physiologically linked to the broadly conserved octadecanoid pathway that regulates phytochemical induction of resistance (Alborn et al., 1997; Thaler, 1999). If the octadecanoid pathway is defective in sweet *C. sativus* plants, then they may also lack induction of volatiles that attract predaceous arthropods.

Why all plants do not constitutively express high levels of plant resistance is still debated (Fritz and Simms, 1992; Mole, 1994; Karban and Baldwin, 1997; Agrawal and Karban, 1999). The current paradigm of allocation based trade-offs has not been strongly supported by experiments that have measured costs of constitutive or inducible defenses (Parker, 1992; Simms, 1992; Karban, 1993; Bergelson and Purrington, 1996; Karban and Baldwin, 1997). Under the conditions of this study, we detected no energetic cost of constitutive or inducible resistance in *C. sativus.* Root and shoot biomass accumulation was not affected by genotypic (bitter or sweet) or induced expression of cucurbitacins and the correlated resistance to spider mites. Cucurbitacins are carbon-based molecules

lacking potentially expensive nitrogen and may even serve the plant in temporary carbon storage. Multiple roles for plant defenses may mask their costs.

Why then might variation in plant resistance be maintained? In this study, we found evidence that induced plant resistance to spider mites was associated with increased feeding by specialist diabroticite beetles. Similar results have been obtained by O. Cheeseman (personal communication), who found that diabroticites were attracted to and fed more on damaged *Cucurbita pepo* plants compared with undamaged control plants. Interestingly, diabroticites will often ignore cucurbits without cucurbitacins (Tallamy and McCloud, 1991). Thus, at the phenotypic level, induction of cucurbitacins is a double-edged sword, defending plants against some herbivores while attracting others. At the genotypic level, resistance to mites associated with susceptibility to diabroticite beetles (and vice versa) has been known as one of the classic examples in trade-offs in plant defense (Chambliss and Jones, 1996; Da Costa and Jones, 1971).

Trade-offs between resistance and susceptibility to generalist and specialist herbivores are not likely to be specific to the Cucurbitaceae. We are currently investigating similar trade-offs between brassicaceous plants and their specialist and generalist lepidopteran herbivores. Interactions between plants in several other families and specialist and generalist herbivores appear to mirror these patterns (Matsuda, 1988; Wink, 1988; Linhart, 1991; Pasteels and Rowell-Rahier, 1992; Vaughn and Hoy, 1993; Landau et al., 1994; Matsuki and MacLean, 1994; Loughrin et al., 1995, 1996; Alados et al., 1996; Jiang et al., 1997; van Dam and Hare, 1998).

Other constraints and selection pressures may also favor constitutive and induced plant variation in resistance traits (reviewed by Agrawal and Karban, 1999). For example, plants may not be able to maximize defense against the many different types of attackers. In *C. sativus*, resistance induced by pathogens is not effective against herbivores (Ajlan and Potter, 1991; Apriyanto and Potter, 1990). These authors found that inoculation of plants with various pathogens led to induction of resistance against subsequent attack by several pathogens. However, these induced responses did not affect performance or preference of a variety of arthropod herbivores including spider mites, aphids, noctuid larvae, and whiteflies. Reciprocal initial attack by arthropods did not affect plant resistance to the pathogens. Evidence from other systems indicates that these independent plant defense responses may be controlled by separate pathways, which may actually inhibit each other (Doares et al., 1995; Thaler et al., 1999). Inducible defenses may be favored to maximize a plant's ability to defend itself against various attackers, since resistance to all attackers at once may not be physiologically possible. It is also possible that molecules such as cucurbitacins serve multiple functions, and selection on one function may be tempered by selection on other functions (Tallamy and Krischik, 1989).

There may be other costs and benefits of variation in plant resistance traits

(Mole, 1994; Karban and Baldwin, 1997; Agrawal and Karban, 1999; Agrawal et al., 1999b,c). For example, Krischik and Tallamy (unpublished) have demonstrated that cucurbitacins enhance the fitness of plants exposed to ultraviolet light (UV-B irradiance). However, expression of compounds implicated in defense may be constrained by physiological problems, such as autotoxicity. Plant resistance against herbivores may also interfere in interactions with mutualists such as seed dispersers and pollinators (Stephenson, 1982; Willmer and Stone, 1997). Many cucurbits have cucurbitacins in their flowers (Andersen and Metcalf, 1987). *Cucumis sativus* fruits with cucurbitacins were visited significantly less often by wasps and honeybees than fruits without cucurbitacins (Da Costa and Jones, 1971). Variation in plant resistance traits may maximize defense in additional ways that we discuss in detail elsewhere (Agrawal and Karban, 1999). It is clear from this and other plant–herbivore studies that to fully understand variable defenses we will have to look beyond the allocation trade-off model.

In *C. sativus*, there appears to be strong genetic variation for constitutive and induced variation in plant resistance to spider mite herbivory. This variation was not correlated with allocations to plant growth. However, high levels of resistance to spider mites associated with high levels of cucurbitacins were correlated with susceptibility to attack by specialist diabroticite beetles. Such ecological trade-offs are likely to be more common than previously thought and may play a role in the maintenance of constitutive and induced variation in plant resistance traits.

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PHEROMONE COMPONENTS AND DIEL PERIODICITY OF PHEROMONAL COMMUNICATION IN *Lymantria fumida*

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Abstract—Extracts of pheromone glands from female *Lymantria fumida* were analyzed by coupled gas chromatographic–electroantennographic detection (GC-EAD) and by coupled GC–mass spectrometry (MS). The two compounds that elicited responses from male *L. fumida* antennae were identified as *cis*-7,8-epoxy-2-methyloctadecane (disparlure) and 2-methyl-*Z*7-octadecene (2me-*Z*7–18Hy). Field experiments in northern Japan demonstrated that synthetic (7*R*,8*S*)-*cis*-7,8-epoxy-2-methyloctadecane [(+)-disparlure] and 2me-*Z*7–18Hy are synergistic sex pheromone components of *L. fumida.* (7*S*,8*R*)-*cis*-7,8-Epoxy-2-methyloctadecane [(−)-disparlure] had no behavioral effect on male *L. fumida.* Traps baited with (+)-disparlure and 2me-*Z*7–18Hy captured male *L. fumida* between 21:00 and 24:00 hr, whereas traps baited with (+)-monachalure [(7*R*,8*S*)-*cis*-7,8-epoxy-octadecane], (+)-disparlure and 2me-*Z*7–18Hy attracted males of the nun moth, *L. monacha* L., between 02:00 and 04:00 hr. Both temporal separation of pheromonal communication and specificity of pheromone blends seem to contribute to the reproductive isolation of sympatric and coseasonal *L. fumida* and *L. monacha.*

Key Words—Lepidoptera, Lymantriidae, *Lymantria fumida*, *Lymantria monacha*, nun moth, sex pheromone, periodicity, calling behavior, reproductive isolation, disparlure, (7*R*,8*S*)-*cis*-7,8-epoxy-2-methyloctadecane, (7*S*,8*R*)-*cis*-7,8-epoxy-2-methyloctadecane, 2-methyl-*Z*7-octadecene, (7*R*,8*S*)-*cis*-7,8-epoxy-octadecane, (+)-monachalure.

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INTRODUCTION

Lymantria fumida Butler (Lepidoptera: Lymantriidae) occurs in Japan (main islands except Hokkaido), eastern China, eastern Russia, and Taiwan (Inoue, 1957). In Japan, it inflicts damage on Japanese fir, *Abies firma* Sieb. et Zucc. (Inoue, 1957) and reached outbreak population levels on larch, *Larix leptolepis* (Sieb. et Zucc.) Gord. in northern Honshu (Sato, 1979). Congeneric gypsy moth, *L. dispar* L., and nun moth, *L. monacha* L., are sympatric with *L. fumida* in many areas of Japan. The sex pheromone of European strains of *L. dispar* [(+)-disparlure = (7*R*,8*S*)-*cis*-7,8-epoxy-2-methyloctadecane (Bierl et al., 1970; Cardé et al., 1977a,b; Miller et al., 1977; Plimmer et al., 1977], and *L. monacha* [(+)-disparlure (Bierl et al., 1975; Klimitzek et al., 1976), (+)-monachalure = (7*R*,8*S*)-*cis*-7,8-epoxy-octadecane (Gries et al., 1996, 1997a), and 2-methyl-*Z*7-octadecene (Grant et al., 1996; Gries et al., 1996)] have been studied, but little is known about the pheromone of *L. fumida*. Capture of male *L. fumida* in traps baited with (+)-disparlure (Beroza et al., 1973) suggested that disparlure could be part of the *L. fumida* sex pheromone. We report identification and field testing of sex pheromone components of *L. fumida* and diel periodicity of captures of male moths in pheromone-baited traps.

METHODS AND MATERIALS

Insect Cultures and Pheromone Analyses. In July 1996, female *L. fumida* were collected from *L. leptolepis* around the Tohoku Research Centre (Morioka) and kept individually in paper bags for oviposition. Eggs, tucked under flaps or folds in the paper, were hand carried to a quarantine facility of the United States Department of Agriculture (USDA) in Newark, Delaware. Well after completion of embryonation, eggs were chilled and overwintered at 2°C. In mid-March, after changing environmental conditions to 25°C, 60–80% relative humidity, and a 14L : 10D photoperiod, eggs hatched. Larvae were kept in open plastic boxes (with a petrolatum jelly around the rim) and fed on bouquets of *L. leptolepis* branches kept in water, which were initially greenhouse-forced into foliation and later field collected. Female pupae (Sato, 1979) were maintained at laboratory conditions as above, whereas male pupae were stored at 13°C to retard development. In early May, female moths emerged. Two to three hours into the scotophase, abdominal tips with pheromone glands of calling, 2- to 3-day-old females were removed and extracted for 15–30 min in HPLC-grade hexane. Ampoules with the supernatant of pheromone extracts and male pupae were hand carried to Simon Fraser University.

Aliquots of 1 female equivalent (FE) of pheromone gland extract were analyzed by coupled gas chromatographic–electroantennographic detection (GC-

EAD) (Arn et al., 1975), using a Hewlett Packard (HP) 5890A gas chromatograph equipped with a fused silica column (30 m × 0.25 or 0.32 mm ID) coated with either DB-210, DB-5, or DB-23 (J&W Scientific, Folsom, California). GC-EAD analyses were replicated five to eight times with each of these fused silica columns. Antennal activity of (+)- and of (−)-disparlure at 1-pg quantities was assessed, using the same male antenna for consecutive GC-EAD analyses of respective standards. GC–mass spectra (MS) of synthetic or insect produced compounds in full scan electron ionization mode were obtained from a Varian Saturn II Ion Trap GC-MS fitted with the DB-23 column referred to above.

Field Experiments. Field experiments were conducted in plantings of mature *L. leptolepis* around Morioka, Iwate Prefecture. Self-made sticky 2-liter Delta-type milk carton traps (Gray et al., 1984) were suspended from trees ~2 m above ground in complete randomized blocks with trap spacings of 15–20 m. Traps were baited with gray sleeve stoppers (The West Company, Lionville, Pennsylvania) impregnated with test chemicals in HPLC-grade hexane. Experiment 1 tested the major candidate pheromone component (+)-disparlure (10 μg) alone and in binary combinations with 2-methyl-*Z*7-octadecene (2me-*Z*7–18Hy) at increasing doses. Experiment 2 tested 2me-*Z*7–18Hy alone and in combination with (+)-disparlure and either or both (+)- and (−)-disparlure. Diel periodicity of pheromonal communication in *L. fumida* and coseasonal *L. monacha* was assessed in experiment 3 by hourly recording captures of male moths in traps baited with the two-component blend of (+)-disparlure (50 μg) and 2me-*Z*7–18Hy (5 μg) for male *L. fumida* or with the three component blend of (+)-monachalure (50 μg), 2me-*Z*7–18Hy (5 μg) and (+)-disparlure (5 μg) for male *L. monacha* (Gries et al., unpublished data).

RESULTS AND DISCUSSION

GC-EAD analyses of pheromone gland extract of female *L. fumida* revealed two compounds that consistently elicited responses from male moth antennae (Figure 1). Retention indices (Dool and Kratz, 1963) of most EAD-active **B** on the three analytical columns (DB-5, DB-210, and DB-23) were identical to those of disparlure. This tentative structural assignment was confirmed by comparative retention, mass spectrometric, and EAD characteristics of insect-produced **B** and synthetic disparlure. EAD-active **A** had retention indices indicative of the olefin analog of disparlure, and through comparative GC-EAD on all three columns was confirmed to be 2me-*Z*7–18Hy. In consecutive GC-EAD analyses of (+)- and of (−)-disparlure at 1 pg each, the same antenna of male *L. fumida* responded well to (+)-disparlure but not to the antipode (Figure 1). In field experiment 1 (Figure 2), (+)-disparlure by itself was hardly attractive to male *L. fumida*, but addition of 2me-*Z*7–18Hy at increasing doses synergistically enhanced attractiveness of the bait. Traps baited with 2me-*Z*7–18Hy in combination with (+)-, (+)- plus (−)-, or

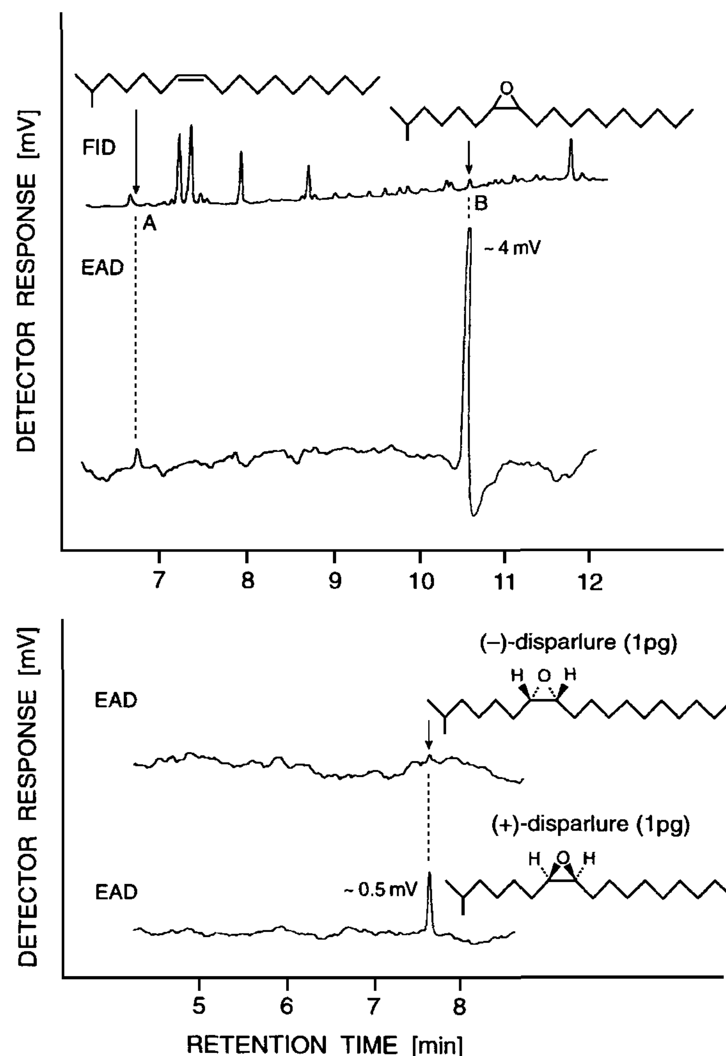

FIG. 1. Top: Flame ionization detector (FID) and electroantennographic detector (EAD: male *L. fumida* antenna) responses to 1 FE of pheromone gland extract. Chromatography: splitless injection; injector and FID detector: 240°C; DB-23 column; temperature program: 100°C (1 min), then 10°C/min to 200°C (5 min). Bottom: Representative GC-EAD recording ($N = 5$) of a male *L. fumida* antenna responding in sequence to separately injected (−)- and (+)-disparlure; chromatography: splitless injection; injector and FID detector: 240°C; DB-23 column; temperature program: 130°C (1 min) then 10°C/min to 200°C (5 min).

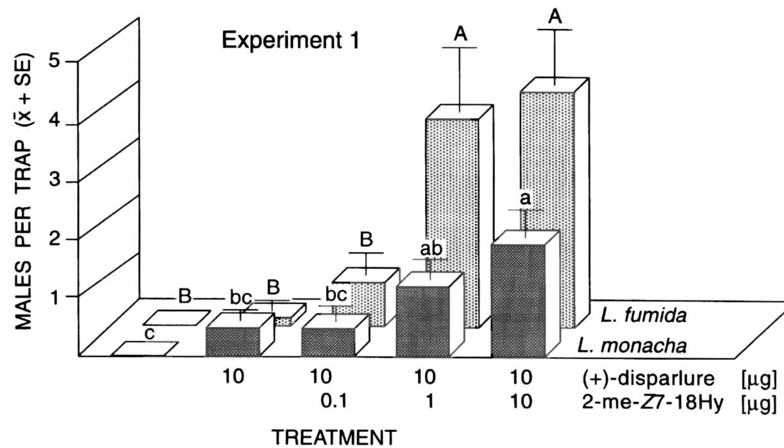

FIG. 2. Captures of male *L. fumida* and *L. monacha* in sticky Delta traps baited with two candidate pheromone components; experimental forest of the Tohoku Research Centre, Morioka, Japan; July 23–25, 1997; eight replicates. Bars within each row with the same superscript letter are not significantly different; nonparametric analysis of variance by ranks (Friedman's test) followed by comparison of means (Student-Newman-Keuls test) (Zar, 1984), $P < 0.05$.

(±)-disparlure captured similar numbers of male *L. fumida*, whereas traps baited with 2me-*Z*7–18Hy singly, or in combination with (−)-disparlure, failed to capture any male moths (Figure 3, experiment 2). Captures of male *L. fumida* and *L. monacha* in pheromone-baited traps occurred between 21:00 and 24:00 hr, and between 02:00 and 04:00 hr, respectively (Figure 4).

Laboratory analyses of pheromone extract and field experiments provide conclusive evidence that (+)-disparlure and 2me-*Z*7–18Hy are synergistic sex pheromone components of *L. fumida.* (−)-Disparlure with only weak antennal activity (Figure 1) does not contribute to the attractiveness of the sex pheromone, although female moths may still release (−)-disparlure to enhance pheromone specificity. European female *L. monacha*, e.g., use (+)-disparlure as a pheromone component, but likely also produce (−)-disparlure (Hansen, 1984), which reduces attraction of European (Cardé et al., 1977a,b; Miller et al., 1977; Plimmer et al., 1977) and northern Japanese (Gries et al., unpublished) male *L. dispar* to (+)-disparlure. Whether 2me-*Z*7–18Hy not only disrupts the response of European male *L. dispar* (Cardé et al., 1973; Miller et al., 1977), but also Asian male *L. dispar* is yet to be investigated. If so, 2me-*Z*7–18Hy is a pheromone component, which—analogous to its role in *L. monacha* (Grant et al., 1996; Gries et al., 1996)—synergistically attracts conspecific male moths wile reducing cross-attraction of heterospecific male *L. dispar.*

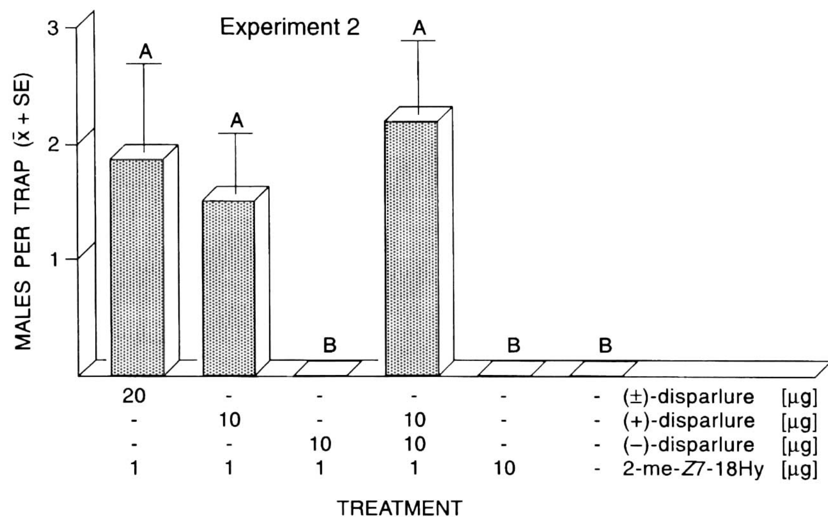

FIG. 3. Captures of male *L. fumida* in sticky Delta traps baited with enantiomers of disparlure or with 2-methyl-*Z*7-octadecene singly and in combinations; experimental forest of the Tohoku Research Centre, Morioka, Japan; July 25–26, 1997; 10 replicates. Bars with the same superscript letter are not significantly different; nonparametric analysis of variance by ranks (Friedman's test) followed by comparison of means (Student-Newman Keul's test) (Zar, 1984), $P < 0.05$.

Response of both male *L. fumida* and male *L. monacha* to (+)-disparlure plus 2me-Z7–18Hy (Figure 2) lets us hypothesize that *L. fumida* and *L. monacha* use temporal separation of pheromonal communication to maintain their reproductive isolation. A shortage of pupae in the field precluded an experiment in which virgin female *L. fumida* and *L. monacha* could have been observed to time their calling behavior and attraction of males. Employing pheromone lures, instead of female moths, for analyzing periodicity of sexual communication seemed justified because periods of pheromone release by females and response by males typically overlap in (lymantriid) moths, as recently demonstrated for the satin moth, *Leucoma salicis* L. (Gries et al., 1997b). Capture of male *L. fumida* between 21:00 and 24:00 hr and male *L. monacha* between 02:00 and 04:00 hr (Figure 4, experiment 3) support the hypothesis that these two congeners employ distinctively different periodicities of pheromone communication. Moreover, the 3-component *L. monacha* blend with (+)-monachalure as the major pheromone component (Gries et al., unpublished) did not attract *L. fumida.*

With accumulating knowledge about pheromone blends and diel periodicities of pheromone communication in lymantriids in northern Japan, including *L. dispar* (Schaefer and Gries, unpublished), *L. fumida* (this study), *L. monacha* (Gries

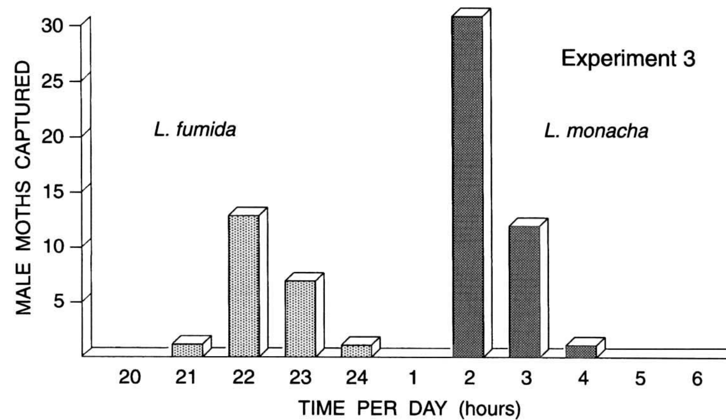

FIG. 4. Representative hourly recording of captures of male *L. fumida* and male *L. monacha* in sticky Delta traps baited with either disparlure (50 μg) and 2-methyl-Z7-octadecene (5 μg) (for *L. fumida*) or with (+)-monachlure (50 μg), (+)-disparlure (5 μg), and 2-methyl-Z7-octadecene (5 μg) (for *L. monacha*); larch forest, Saezurhuo-Sato Bird Sanctuary, ca. 20 km west of Morrioka, Japan, night of July 19–20, 1998. Note: (1) traps with baits for *L. fumida* (N = 10) or *L. monacha* (N = 10) were alternated at 15 to 20-m intervals; (2) there was no cross-attraction of male *L. fumida* to *L. monacha* baits and vice versa; (3) captures of male *L. fumida* between 20:00 and 24:00 hr and of male *L. monacha* between 01:00 and 04:00 hr has been confirmed in three other experiments with varying experimental design and trap baits.

et al., unpublished), and pink gypsy moth, *L. mathura* Moore (Gries et al., 1999), it will now be intriguing to investigate whether, and to what extend, pheromone blends and calling periods vary in accordance with the composition of island-isolated lymantriid communities.

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ELECTROANTENNOGRAM RESPONSES OF A PREDATOR, *Perillus bioculatus*, AND ITS PREY, *Leptinotarsa decemlineata*, TO PLANT VOLATILES

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Abstract—The two-spotted stinkbug, *Perillus bioculatus*, is a predator of the Colorado potato beetle (CPB), *Leptinotarsa decemlineata*. Behavioral tests revealed that *P. bioculatus* is attracted to potato plants, *Solanum tuberosum* L. (Solanaceae), infested by the CPB. Electroantennograms from the antennae of *P. bioculatus* were recorded in response to compounds present in the headspace of CPB-infested potato plants. (*Z*)-3-Hexen-1-ol and 2-phenylethanol elicited the highest EAG amplitudes. Linalool, 4,8-dimethyl-1,3(*E*),7-nonatriene, nonanal, decanal, and (*R*)-(+)-limonene evoked lower EAG amplitudes. The major headspace components β-caryophyllene and β-selinene produced only weak EAG responses. Antennal sensitivity of the CPB to (*Z*)-3-hexen-1-ol was higher than that of *P. bioculatus*, whereas the stinkburg was more sensitive to 2-phenylethanol, β-caryophyllene, (*R*)-(+)-limonene, and decanal. Among these compounds, 2-phenylethanol is of special interest since it was observed to be emitted by potato foliage only after being damaged by CPBs.

Key Words—Hemiptera, Pentatomidae, *Perillus bioculatus*, Coleoptera, Chrysomelidae, *Leptinotarsa decemlineata*, electroantennography, EAG, tritrophic interactions, infochemicals, synomones, plant volatiles.

INTRODUCTION

Perillus bioculatus (Fabr.) is a well-known predator of the Colorado potato beetle, *Leptinotarsa decemlineata* (Say), (CPB), one of the most devastating insect pests in potato cultures. Although this stinkbug can be reared on diverse prey

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species, it seems to feed nearly exclusively on the CPB under field conditions (Saint-Cyr and Cloutier, 1996, and references therein). The use of natural enemies in biological control of the CPB has been discussed since its first appearance in cultivated potatoes in 1860 (Casagrande, 1987). However, establishment of *P. bioculatus* in Europe could not be achieved (Franz, 1966; Bjegovic, 1970). Nevertheless, a successful control of the CPB is possible, when great numbers of the stinkbug are released in potato fields infested by the CPB (Hough-Goldstein and Keil, 1991; Biever and Chauvin, 1992; Cloutier and Bauduin, 1995). The extent to which *P. bioculatus* is able to orient to volatiles derived from its prey is controversial. In his review, Jermy (1980) pointed out that previous tests revealed neither an affinity of *P. bioculatus* for potato plants nor a sexual attraction to mating partners. Furthermore, a distant detection of prey could not be demonstrated (Franz, 1967). Meanwhile, hints on sex specific volatiles with a possible role as pheromones were given by Aldrich et al. (1986). A first hint on distant prey detection was provided by the observation of behavioral changes of *P. bioculatus* shortly before a prey is encountered (Heimpel and Hough-Goldstein, 1994). Van Loon et al. (1999) demonstrated that *P. bioculatus* is attracted towards volatiles emitted by potato plants infested by the CPB. The volatiles emitted by mechanically damaged plants also attracted *P. bioculatus*. However, this effect was observed only within 1 hr after damaging the plants. Plants infested by larvae of the CPB remained attractive for a longer period, even after removal of the larvae. The attraction of a predator to plants infested by a phytophagous arthropod is a phenomenon first observed for predatory mites, which use spider mite-induced plant odors for detection of their prey (Dicke and Sabelis, 1988; Dicke et al., 1990). Interestingly, the CPB, too, is attracted to potato plants already infested by conspecifics (Bolter et al., 1997; Schütz et al., 1997). However, the extent to which CPB and *P. bioculatus* use the same volatiles is unknown.

A first step to identify substances relevant for the orientation of *P. bioculatus* can be taken by using electrophysiological methods. Electroantennography (EAG) is a method for recording of slow potentials from the antennae of insects (Schneider, 1957). After stimulation of the antenna with adequate stimuli, the recorded potential shows a deflection and returns to the prestimulation level within a few seconds. The amplitude of the deflection and the restoration time depend on quality and quantity of the odor used for stimulation. The response obtained reflects a summation over receptor potentials elicited in many olfactory cells.

Few results of EAG experiments with pentatomid bugs have been published. A study with the phytophagous species *Nezara viridula* revealed that this bug responds to a variety of plant volatiles (Pavis and Renou, 1990). EAG experiments with the predaceous bugs *Podisus maculiventris* and *P. nigrispinus* (Sant'Ana and Dickens, 1998) document an olfactory sensitivity of both preda-

tory bugs for a range of compounds comprising green leaf volatiles, monoterpenes, aldehydes, and some others. These volatiles might be used for detection of their phytophagous prey but partly also play a role as aggregation pheromones (Aldrich et al., 1984). Although data on the EAG responses of the CPB to plant volatiles have been published (Visser, 1979; Weissbecker et al., 1997b), additional experiments with the CPB were performed in this study to allow a direct comparison of the sensitivity of both insects to certain plant odors.

Several recent publications present information on the substances emitted from potato plants after infestation by the CPB (Bolter et al., 1997; Schütz et al., 1997; Weissbecker et al., 1997a). Some of the substances identified were emitted in considerably increased amounts when the potato plants were damaged by the CPB [e.g., 2-phenylethanol, nonanal, and 4,8-dimethyl-1,3(*E*),7-nonatriene], while others [e.g., (*Z*)-3-hexen-1-ol, limonene, decanal, and caryophyllene] were also emitted after mechanical damage or even by undamaged plants. The behavioral responses of *P. bioculatus* towards plants infested by the CPB observed by van Loon et al. (1998) suggest that some of the emitted plant odors are perceived by the stinkbug. To test this hypothesis, EAG signals in response to these volatiles were recorded in this study.

METHODS AND MATERIALS

Plants and Insects. Potato plants (cv. Surprise) were grown in a greenhouse at 21 ± 2°C and a 16-hr photoperiod. Artificial daylight was supplemented with 400-W high-pressure sodium-vapor lamps hanging 1 m above pot level when natural light intensity fell below 240 W/m^2.

CPBs were reared on excised potato foliage at 20–25°C and a 16-hr photoperiod. For pupation the fourth-instars were placed in boxes filled with sand. Adult beetles were collected on the day when they emerged from the sand and were fed with potato foliage for 7–9 days. Prior to EAG experiments, adult CPBs were deprived of food for 4–6 hr. During the starvation period CPBs were kept in Petri dishes with moistened filter paper to prevent desiccation of the beetles.

Two-spotted stinkbugs were reared on larvae of the CPB under the same temperature and photoperiod as described above. In the rearing cages the nymphs and adults of the stinkbug also had access to cotton wool drenched with water and to potato plant material. The bottom of each cage was covered with pieces of polystyrene in order to provide shelter for the stinkbugs. Prior to experiments, the stinkbugs were preconditioned in the following way (van Loon et al., 1999): Freshly molted stinkbug adults were kept individually in Petri dishes (9 cm ID) provided with moistened filter paper and some cotton wool soaked with water. An additional folded piece of filter paper provided a shelter for the stinkbugs. In the first 24 hr of the conditioning period, the stinkbugs had no access to food.

For the following 24 hr each stinkbug was provided with three fourth-instars of the CPB, thus having food ad libitum. The next 24 hr were again a starvation period.

Odor Standards and Stimulus Delivery. Odor standards were prepared by diluting pure compounds in paraffin oil (Uvasol, Merck, Darmstadt, Germany). For each compound to be tested in the EAG setup, a dilution series was prepared starting with a 10^{-1} (w/w) dilution followed by further 1 : 10 dilution steps. The following chemicals were used: (Z)-3-hexen-1-ol, 2-phenylethanol, (*R*)-(+)-limonene, nonanal, β-caryophyllene (all from Sigma-Aldrich, Steinheim, Germany), linalool (Roth, Karlsruhe, Germany), and decanal (Fluka, Buchs, Switzerland). The purity of the commercially available chemicals ranged from 95 to 99%. 4,8-Dimethyl-1,3(*E*),7-nonatriene and β-selinene were provided by the Department of Organic Chemistry (Wageningen Agricultural University). To synthesize 4,8-dimethyl-1,3(*E*),7-nonatriene (purity ~85%) geraniol was oxidized with CrO_3 pyridine to geranial, which was converted to the desired product with the Wittig reagent methyltriphenylphosphonium iodide. The sesquiterpene β-selinene (purity ~97%) was isolated from celery oil (*Apium graveolens*). Because of the poor solubility of 2-phenylethanol in paraffin oil, the dilution series of this compound started with the 10^{-2} dilution. Because β-selinene was available in very small amounts, only a 10^{-2} dilution was prepared. Filter papers (6 × 0.5 cm) were soaked with 25 μl of the standard solutions and put in Pasteur pipets. The odor concentration in the air contained by the pipets is proportional to the concentration of the compound in the solution (Raoult's law). The pipets were inserted in the stimulus delivery system (model CS-01/b, Syntech, Hilversum, The Netherlands). This system provided puffs of air with defined volume and duration (ca. 1.75 ml in 0.5 sec) and simultaneously triggered the data acquisition system. The puff of air loaded with the odor substances was introduced into a stronger stream of charcoal-filtered humidified air (900–1000 ml/min) that was flowing over the antennal preparation at a velocity of 20 cm/sec. Each odor source was used for a maximum of 10 injections and then discarded.

Preparation of Insects. EAG recordings were made from intact insects. Holders for CPBs and stinkbugs were made of Plexiglas blocks with a rectangularly shaped excavation that was broad enough to house the respective insect. For immobilisation of the stinkbug, the holder was lined with a double-sided adhesive tape. Then the bugs were placed dorsum down into the excavation with legs stretched on the heightened parts of the holder. The holder was wrapped with a strip of Parafilm so that only the head of the bug remained free. One antenna of the bug was loosely fixed on the sticky tape and the tip was cut off with a scalpel. An opening for the reference electrode was cut into the head of the bug with a pair of microscissors. After insertion of the recording electrode, the antenna was lifted from the adhesive tape in order to allow the airstream to flow freely around the antenna. The CPB had to be tied more thoroughly. In

order to compensate for the rounded shape of the beetle, the excavation of the holder was lined with plasticine. The holder was wrapped with Parafilm while the beetle's legs were drawn up to its body. Again, only the head of the insect remained free. After pricking a small hole in the right eye of the beetle for the insertion of the reference electrode, another strip of Parafilm was twisted to a string and wrapped around the holder longitudinally. This string lodged between the mandibles of the beetle and prevented the beetle from moving its head. A small drop of dentist's wax, applied with a Pasteur pipet, fixed the base of the left antenna to the head of the beetle. At this stage, the tip of the antenna was cut off with a pair of microscissors.

EAG Recording and Procedure of Measurements. Recording and indifferent electrodes were Ag–AgCl electrodes in a saline solution (NaCl: 154 mM, KCl: 26.8 mM, $CaCl_2$: 9.1 mM) to which polyvinylpyrrolidon (5 g/liter) was added. The glass capillaries (1.5 mm OD) were filled with saline. Amplifying and recording of the EAG signals were done using a headstage amplifier, a "signal interface box" (model ID-04), and a PC-based data acquisition system (call from Syntech, Hilversum, The Netherlands).

Stimulations were made at intervals of 1 min beginning with the lowest concentration of the compounds and ending with the highest concentration. CPBs were stimulated either with a dilution series of (Z)-3-hexen-1-ol or with the eight compounds listed in Table 1. Up to four dilution series of different compounds were assayed on each stinkbug. Additional injections were made with a reference standard [10^{-3} w/w (Z)-3-hexen-1-ol] and controls (paraffin oil). Thus, depending on the range of the dilution series, the total number of sample injections per insect ranged from 20 to 30. The sequence of the different compounds was altered from insect to insect. The number of replications for each experiment was 11 or 12.

In the following, the term "EAG response" will be used for the maximum deflection of the recorded EAG signal after stimulation with an odor. For the comparison of the sensitivity of *P. bioculatus* and the CPB for different plant volatiles, all recorded EAG responses are expressed as a percentage of the response to the reference standard. This expression allows for compensation for changes of the sensitivity of an antenna during the course of an experiment. Furthermore, a comparison between experiments performed with antennae of different sensitivities is possible.

RESULTS

EAG Responses of P. bioculatus. Among all odor substances tested, (Z)-3-hexen-1-ol evoked the highest EAG responses from *P. bioculatus.* Typical EAG recordings obtained after stimulation with this green-leaf-volatile are depicted

TABLE 1. COMPARISON OF SCALED RESPONSES (AVERAGE ± SD) OF *P. bioculatus* AND COLORADO POTATO BEETLE TO PLANT ODORS[a]

Stimulus		EAG response		U test
Compound	Dilution	*P. bioculatus*	CPB	(betw. species)
Paraffin oil		19.9 ± 6.0	23.0 ± 6.5	NS
(*R*)-(+)-Limonene	10^{-3}	33.2 ± 5.8[b]	25.5 ± 7.6	**
Decanal	10^{-3}	34.7 ± 9.2[b]	25.6 ± 6.0	**
Nonanal	10^{-3}	37.8 ± 10.2[b]	47.7 ± 8.7[c]	*
4,8-Dimethyl-1,3(*E*),7-nonatriene	10^{-3}	47.2 ± 6.9[b]	40.1 ± 8.9[c]	NS
Linalool	10^{-3}	53.9 ± 8.7[b]	58.3 ± 12.9[c]	NS
2-Phenylethanol	10^{-3}	97.3 ± 17.4[b]	59.2 ± 10.2[c]	***
β-Caryophyllene	10^{-2}	36.0 ± 6.1[b]	25.4 ± 6.4	**
β-Selinene	10^{-2}	36.8 ± 8.8[b]	35.7 ± 7.4[c]	NS

[a]Last column shows significance levels for differences between species. Mann-Whitney U test (***$P < 0.001$; **$0.001 \leq P < 0.01$; *$0.01 \leq P < 0.05$; NS: $0.05 \leq P$).
[b]EAG response significantly different from paraffin oil (Wilcoxon matched pair signed-ranks test, $\alpha = 0.05$). For each compound, a separate paraffin oil control was measured with the same set of antennae. The values for each compound are statistically tested versus their respective controls. The paraffin oil value in the table is the average of all paraffin oil measurements together.
[c]EAG response significantly different from paraffin oil (Wilcoxon matched pair signed-ranks test with Bonferroni correction, $\alpha = 0.0063$).

in Figure 1. The other plant odors caused responses of similar shape, but with smaller amplitudes. *Perillus bioculatus* antennae displayed a relatively fast recovery of sensitivity after stimulation with high doses of the odors. In general, the response of *P. bioculatus* to the reference standard remained constant during

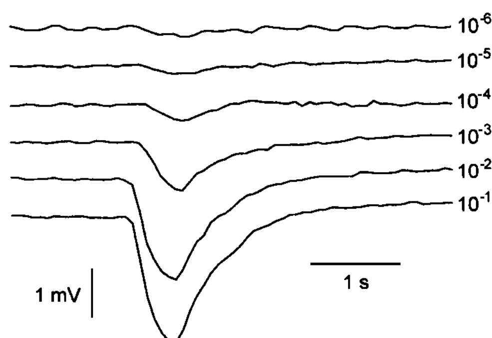

FIG. 1. Typical EAG responses recorded successively from an individual *P. bioculatus* after stimulation with a concentration series of (Z)-3-hexen-1-ol. Numbers at the EAG tracks indicate dilution (w/w) in paraffin oil.

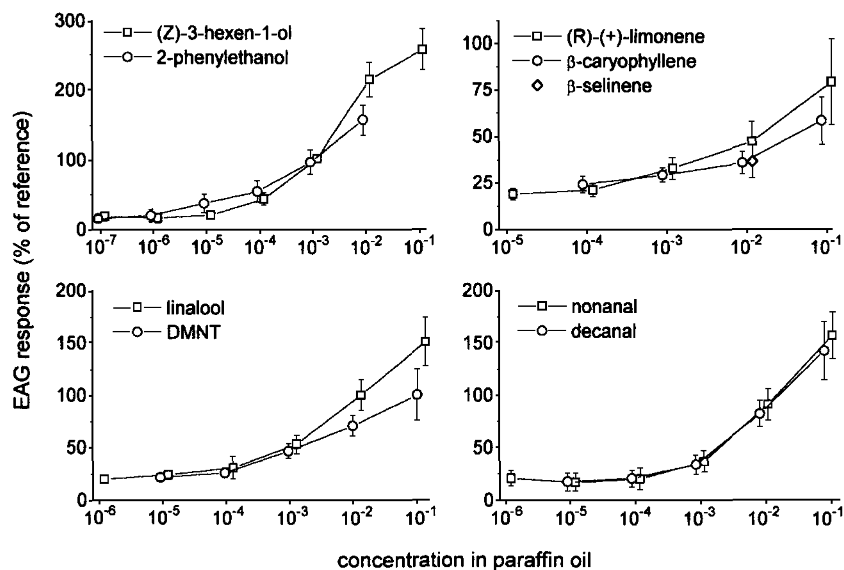

FIG. 2. Dose–response relationships for stimulation of *P. bioculatus* antennae with plant odors [DMNT = 4,8-dimethyl-1,3(*E*),7-nonatriene]. Each data point represents the average of the scaled responses of 11 or 12 different antennae. Standard deviations are indicated as error bars. Data points are slightly shifted in the position on the x-axis in order to avoid overlap of the error bars. The responses are scaled with the response to the reference standard.

measurements even after application of stimuli that evoked high EAG responses. The dose–response characteristics of *P. bioculatus* to the tested dilution series are depicted in Figure 2. In the whole set of experiments the EAG response to the reference [10^{-3} w/w (Z)-3-hexen-1-ol in paraffin oil] ranged from 0.22 mV to 1.45 mV with an average of 0.84 ± 0.26 mV. The sex of the bugs was recorded in all experiments. Significant (Mann-Whitney U test, $P < 0.05$) sex-specific differences in the sensitivity to the plant odors were observed only for two of the 45 standards (2-phenylethanol in 10^{-6} dilution and (*R*)-(+)-limonene in 10^{-2} dilution). Therefore, the data obtained with female and male bugs were pooled. (Z)-3-Hexen-1-ol and 2-phenylethanol elicited in the highest EAG responses of *P. bioculatus*. At the 10^{-3} dilution level the responses to these compounds were not significantly different from each other (Mann-Whitney U test, $P > 0.05$), but significantly higher than the responses to all other compounds (Mann-Whitney U test, $P < 0.001$). At low dilution levels (10^{-5} and 10^{-4}) the responses to 2-phenylethanol were higher than to (Z)-3-hexen-1-ol (Mann-Whitney U test, $P < 0.05$). This is remarkable since the vapor pressure of 2-phenylethanol is lower

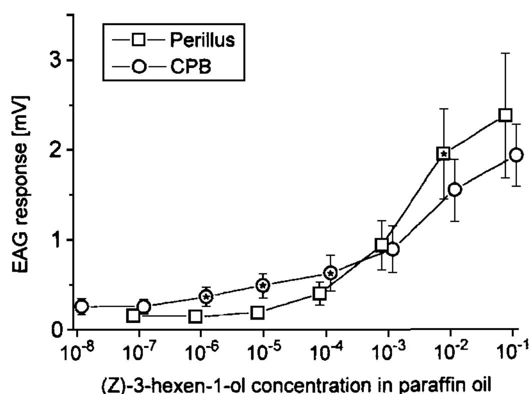

FIG. 3. Comparison of the sensitivity of *P. bioculatus* and the CPB for (Z)-3-hexen-1-ol. Each data point represents the average of the absolute responses of 11 or 12 different antennae. Standard deviations are indicated as error bars. Data points are slightly shifted in their position on the x axis in order to avoid overlap of the error bars. Asterisks indicate if differences in the responses between species are significant (Mann-Whitney U test, $P < 0.05$).

than that of (Z)-3-hexen-1-ol, so that the number of molecules delivered with the stimulus is lower for 2-phenylethanol. The low responses to the sesquiterpenes β-caryophyllene and β-selinene correlate with the low vapor pressures of these compounds. In contrast, (*R*)-(+)-limonene has a comparably high vapor pressure, but the responses to this compound were not much higher than to the sesquiterpenes. At the 10^{-2} dilution level, the responses to linalool, nonanal, and decanal do not differ much from each other, which correlates with the similarity of the vapor pressures of these compounds.

EAG Responses of the CPB. No sex specific differences in the EAG recordings from the CPB were observed. To compare the olfactory sensitivity of *P. bioculatus* to that of the CPB, the EAG responses of both insects to a (*Z*)-3-hexen-1-ol dilution series have been recorded (Figure 3). Figure 3 depicts the averages of the absolute EAG responses of both insect species to the compound used as reference standard. Detection limits were estimated by fitting logistic curves to the dose–response data. The concentrations where confidence intervals ($\alpha = 0.05$) of the average control responses did not overlap with the confidence belts of the fit functions were regarded as the limits of detection. For the CPB the threshold concentration was 1.5×10^{-6}, while a concentration of 2×10^{-5} is needed for evoking significant responses from *P. bioculatus*. A comparison of the sensitivity between the tested species indicates a more than 10-fold higher sensitivity of the CPB for (Z)-3-hexen-1-ol as compared to *P. bioculatus.*

The rise in the dose–response characteristics of *P. bioculatus* is steeper than

that of the CPB, so that the dose–response curves cross. For the 10^{-3} concentration of (Z)-3-hexen-1-ol both insects show similar responses (*P. bioculatus:* 0.93 ± 0.27 mV, CPB: 0.89 ± 0.26 mV). For concentrations above 10^{-3}, the responses of *P. bioculatus* are slightly higher than that of the CPB. Significant differences (Mann-Whitney U test, $P < 0.05$) in the responses are indicated in Figure 3.

In Table 1 the scaled EAG responses to a series of compounds recorded from *P. bioculatus* and the CPB are compared. *Perillus bioculatus* responded to all standard odors with a significantly higher EAG signal as compared to the control (Wilcoxon, matched pairs signed ranks test, $P < 0.05$). In contrast, the EAG signals of the CPB to β-caryophyllene, (*R*)-(+)-limonene, and decanal were not significantly above the control (Wilcoxon matched pairs signed ranks test, $P < 0.00625$, with Bonferroni correction since all tested compounds share the same control). For these three compounds and for phenylethanol, the EAG responses differ significantly (Mann-Whitney U test, $P < 0.01$) between the two insect species.

DISCUSSION

Perillus bioculatus shows significant EAG responses to a range of plant volatiles that are emitted by potato plants after mechanical damage or damage induced by CPBs. From all substances tested in this study, the highest responses were observed for (Z)-3-hexen-1-ol. This substance is also known to be detected by the CPB with a very high sensitivity (Visser, 1979). (Z)-3-Hexen-1-ol is also emitted by undamaged potato plants or, in higher amounts, by potato plants after mechanical damage (Schütz et al., 1997). 2-Phenylethanol was emitted by potato plants specifically after damage induced by CPBs (Schütz et al., 1997). Here it was demonstrated that *P. bioculatus* shows high olfactory sensitivity to this compound. The fact that 2-phenylethanol was not recorded in the study of Bolter et al. (1997) might be explained by differences in the cultivars and in the sampling techniques used in the experiments. 2-Phenylethanol is a volatile typical for the aroma of flowers (Surburg et al., 1993) but was also detected in the leaves of tomato plants (*Lycopersicon esculentum*) (Buttery et al., 1987), a plant closely related to the potato.

Another compound emitted by potato plants after infestation by CPBs in considerably higher amounts than by mechanical damage is nonanal (Schütz et al., 1997). This aldehyde is detected by the antennae of *P. bioculatus* with moderate sensitivity comparable to that for decanal.

4,8-Dimethyl-1,3(*E*),7-nonatriene and 1,4,8-trimethyl-1,3(*E*),7(*E*),11-decatetraene are homoterpenes that were observed to be emitted from potato plants after damage induced by the CPB (Bolter et al., 1997). Both compounds were also observed in other plants after infestation by herbivores (Dicke, 1994). *Per-*

illus bioculatus detects 4,8-dimethyl-1,3(*E*),7-nonatriene with a moderate sensitivity, slightly lower than that for linalool, decanal, and nonanal. Nevertheless, even unspecific compounds can be used for the detection of host plants when the insect is capable of evaluating the relative amounts of several odors in a mixture. The insect, in this way, could be able to recognize a specific mixture of unspecific plant odors. Such a situation was observed for the CPB, which shows a change in its attraction to potato plants when the ratio of one of the constituents of the plant odor is altered artificially (Visser and Avé, 1978; Schütz et al., 1997).

The sesquiterpenes β-caryophyllene and β-selinene induced only small EAG responses by *P. bioculatus*. However, it should be noted that the vapor pressures of the sesquiterpenes are far below those of other plant volatiles, e.g., (Z)-3-hexen-1-ol. Thus, a paraffin dilution of a sesquiterpene emits considerably fewer odor molecules than the same dilution of (Z)-3-hexen-1-ol. In fact, potato plants emit sesquiterpenes in far higher quantities than any other plant volatiles. In plants damaged by the CPB, the ratio of the emission of caryophyllene relative to the emission of (Z)-3-hexen-1-ol ranges from 10 : 1 to 100 : 1, depending on the time after infestation (Bolter et al., 1997; Schütz et al., 1997). Thus, the small EAG signals of *P. bioculatus* recorded in response to the sesquiterpenes still leave the possibility open that the stinkbug uses these substances for the detection of potato plants infested by the CPB. An attraction of CPBs to potato odors with an artificially elevated ratio of caryophyllene has been demonstrated by Schütz et al. (1997).

Unfortunately, not all of the substances mentioned in the publications of Bolter et al. (1997) and Schütz et al. (1997) are commercially available. Some sesquiterpenes (e.g., germacrene-D, cardinene) are emitted by CPB-infested potato plants in considerable amounts. In order to test if some of the unavailable compounds are detectable by the stinkbug, electroantennographic detection should be used in combination with a gas chromatographic separation (GC-EAG) (Arn et al., 1975). This method can provide information on the ability of an insect to detect compounds that are not available as standard solutions or that are emitted from plants in amounts too low for a identification by GC-MS.

The results of the comparative experiments with the CPB revealed that the detection threshold of the CPB for (Z)-3-hexen-1-ol is lower than that of *P. bioculatus*. In contrast, *P. bioculatus* detects 2-phenylethanol, (*R*)-(+)-limonene, decanal, and β-caryophyllene at lower concentrations than the CPB. Thus, *P. bioculatus* might be able to get more detailed information on the state of a plant by the sensitive detection of volatiles that are typical of certain kinds of damage.

The high sensitivity of *P. bioculatus* for 2-phenylethanol might be an indicator of the relevance of this compound for prey location of the stinkbug. However, the attraction of *P. bioculatus* to plants after infestation with CPB larvae might also be explained by the prolonged emission of volatiles that also occurs after mechanical damage. Sesquiterpenes continue to be emitted from potato plants

for 12–24 hr both after mechanical damage and damage induced by feeding CPB larvae. Green-leaf volatiles are emitted from potato plants in higher amounts if the plant is damaged mechanically as compared to damage induced by feeding larvae (Schütz et al., 1997). Thus, it might be possible that the attractive effect of mechanically damaged plants is based on the combination of sesquiterpenes plus green leaf volatiles, whereas the attractive effect of plants damaged by CPB larvae is based on the combination of sesquiterpenes plus 2-phenylethanol.

Information on the attractancy of chemicals to an insect can be gained only by behavioral studies. The next step is to search for a synthetic mixture with the smallest possible number of constituents that possesses the same attractancy to *P. bioculatus* as potato plants damaged by CPBs. In this connection the data from EAG experiments can be used as a prescreening of those compounds that might induce behavioral responses in the insects.

The development of a synthetic multicomponent plant synomone that attracts the stinkbug *P. bioculatus* is desirable and could play a role in biological plant protection. A synthetic pheromone for *Podisus maculiventris* is already available and was successfully used for trapping this stinkbug and its nymphs in the field (Aldrich et al., 1984; Sant'Ana et al., 1997). A possible application of these attractants is the trapping of wild individuals in the field for the mass rearing of young predators that can be used for augmentative biological control (Aldrich, 1998). Additional applications might be possible in the manipulation of predator populations in the field in order to enhance the effectivity of biological control measures.

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A ROCK-IRON-SOLUBILIZING COMPOUND FROM ROOT EXUDATES OF *Tephrosia purpurea*

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Abstract—Root exudates from *Tephrosia* plants contained a rock-iron [$Fe(OH)_3$] -solubilizing compound. This was purified to apparent homogeneity and appears to possess an iron-binding phenolic moiety linked to a proteinaceous component that may act as a receptor. The lyophilized purified fraction showed maximum $Fe(OH)_3$-solubilizing activity at 50°C under acidic pH. Its activity was influenced by trace ions (Co, Cu, Zn, B, Mo, Mn) and toxic metal ions (Ni, Al, Pb, Cr, Hg), but ions such as Na, K, Mg, and Ca had no significant effects on its $Fe(OH)_3$-solubilizing activity.

Key Words—*Tephrosia purpurea*, root exudates, $Fe(OH)_3$-solubilizing compound, phytosiderophore, bioremediator.

INTRODUCTION

Iron is a trace element essential for the growth of almost all forms of life; however, its availability is limited to aerobic organisms because of its tendency to form insoluble complexes at biological pH (Ochsner et al., 1995; Powell et al., 1980). A number of adaptive strategies have been evolved by biota to overcome iron-deficiency stress. Production of siderophores under iron stress conditions by microorganisms is well documented (Geurinot, 1994; Briat, 1992; Crichton and Charleteaux-Wauters, 1987; Neilands and Leong, 1986; Neilands, 1981).

Plants have been categorized as having strategy I and strategy II responses based upon their adaptations to iron-deficiency stress (Jolley et al., 1996; Hopkins et al., 1992a; Romheld and Marschner, 1986). Dicotyledenous and nongram-

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inaceous monocot plants make iron available with one or a combination of the following strategy I response mechanisms: decreasing rhizosphere pH by proton extrusion, reduction of iron by extrusion of reductants, reduction of iron at the root plasmalemma, or use of internal chelating compounds. Graminaceous monocots display the strategy II iron stress response mechanism by releasing phytosiderophore (Hansen et al., 1996; Hopkins et al., 1992a; Gries and Runge, 1992; Romheld and Marschner, 1986). However, there is a controversy regarding the proposed grouping of plants based on their iron-deficiency stress responses (Geurinot and Yi, 1994; Marschner and Romheld, 1994; Hopkins et al., 1992b; Lytle and Jolley, 1991).

One approach to solving this issue is to evaluate taxa belonging to different major taxonomic groups for their iron-deficiency stress responses. Such screening of flowering plants may also yield agrochemicals that could serve as soil amendments that promote plant growth in iron-stressed habitats. In this paper, we report the presence of a siderophore-like compound in the root exudates of *Tephrosia purpurea* growing in iron stressed lateritic soils rich in goethite (FeOOH).

METHODS AND MATERIALS

Collection of Root Exudate. Fresh intact roots of *Tephrosia* field-collected plants were washed thoroughly, rinsed with distilled water (DW), soaked for 72 hr in deionized DW, and subjected to gentle shaking. Silver oxide (10 mg/liter) was added to control microbial activity. After 72 hr, exudates from the roots were collected and filtered through Whatman No. 1 filter paper. The filtrate obtained was designated as root exudate. *Tephrosia* plants with intact roots were also tested for siderophore release under no-iron-stress conditions by the addition of 100 μm $FeCl_3$ in deionized DW.

Assay for $Fe(OH)_3$ Solubilization. For the evaluation of Fe-solubilizing activity of the root exudates, the procedure used by Takagi (1976) was employed with little modification. To 10 ml of the sample solution, 0.5 ml of 0.5 M sodium acetate buffer, pH 5.6, and 10 mg of $Fe(OH)_3$ were added. The mixture was allowed to stand with occasional stirring at 55°C for 2 hr. Finally, the reaction mixture was filtered through Whatman No. 1 filter paper in 0.2 ml of 3 N H_2SO_4. For the colorimetric determination of the soluble Fe, 0.5 ml of 8% hydroxlamine hydrochloride was added and was kept for 20 min at 55°C. Subsequently, 0.5 ml of 0.25% *O*-phenanthroline and 1 ml of sodium acetate buffer (2 M, pH 4.7) were added successively. Absorbance was measured at 508 nm.

Purification of $Fe(OH)_3$-Solubilizing Compound. In order to fractionate the active compound from the root exudates, a concentrated exudate was passed through a column (1.2 × 10 cm) of Dowex 1 Cl^- at a flow rate of 1.0 ml/6.0 min. The effluent was collected as the unbound fraction. The column was washed

with 100 ml of DW, and the fraction eluted with 0.1 N HCl was discarded. The Dowex I Cl^- fraction, which eluted between 0.1 N and 0.2 N HCl, was collected and lyophilized by using a Martin Christ β lyophilizer. The lyophilized compound was assayed for $Fe(OH)_3$-solubilizing activity by using Chrome Azurol S reagent.

HPLC. The Dowex I Cl^- fraction obtained in the above step was subjected to analysis at 254 nm in the Waters Photo Diode Array Detector (model 991) -based binary gradient HPLC system by eluting a μ-Bundapacic C_{18} (3.9×300 mm) column with methanol–water (80 : 20) at a flow rate of 0.5 ml/min.

λ_{max}: The λ_{max} of the purified fraction was scanned with a Gilford Spectrophotometer.

Hemagglutination Assay. Hemagglutination activity of the compound was assayed by using rabbit erythrocytes (R-RBC). Blood was collected in autoclaved Alsever's solution (dextrose 2.05%, sodium citrate 0.80%, sodium chloride 0.42%, pH 6.1, adjusted with 10% citric acid before autoclaving at 10 lb for 15 min.) in 1 : 1 ratio. Erythrocytes were washed three times by low-speed centrifugation with 50 mM phosphate buffered saline, pH 7.0 (PBS) and suspended in PBS to make a 5% RBC solution. The Dowex I Cl^- fraction (50 μl) was mixed with an equal volume of 5% erythrocytes, and incubated at 30°C for 20 min. Agglutination was observed under the microscope.

Estimation of Protein. To estimate the protein content in the purified compound, the Dowex I Cl^- fraction (1 mg) was dissolved in 100 μl of 20 mM Tris-Cl^-, pH 7.6. This solution was used for the determination of protein content by following the method of Bradford (1976).

Effect of Various Elements on Fe-Solubilizing Activity of the Compound. To see the effect of the various elements on Fe-solubilization activity of the Dowex I Cl^- fraction, 10 mg of each ion listed in Tables 1 and 2 were added to the sample solution separately prior to the addition of 10 mg $Fe(OH)_3$. This mixture was then subjected to normal $Fe(OH)_3$ solubilization assay as mentioned above. Absorbance was taken at 508 nm.

Treatment of Dowex I Cl^- Fraction with Chrome Azurol S Dye. To confirm the siderophoric nature of the compound, different concentrations of the Dowex I Cl^- fraction were incubated with Chrome Azurol S dye at 25°C for 10 min by following the procedure of Schwyn and Neilands (1987). After 10 min, the absorbance was measured at 630 nm on a Gilford Spectrophotometer. The decrease in absorbance was plotted against compound concentration.

RESULTS AND DISCUSSION

Tephrosia is a perennial dominant species of the herbaceous community in the degraded lands rich in ferric hydroxide/oxyhydroxide; it often forms pure patches on rocky habitats with skeletal soils. Fresh intact roots were used to

TABLE 1. EFFECT OF TRACE AND TOXIC METAL IONS ON $Fe(OH)_3$ SOLUBILIZATION ACTIVITY OF COMPOUND ISOLATED FROM *Tephrosia*[a]

S. No.	Treatment	$Fe(OH)_3$ solublized (μg)	Percent inhibition of $Fe(OH)_3$ solubilization activity
1	CS + $Fe(OH)_3$ (control)	8	0
2	CS + $Al_2(SO_4)_3$ + $Fe(OH)_3$	0.8	90
3	CS + $NiCl_2$ + $Fe(OH)_3$	ppt. formed	ppt. formed
4	CS + MoO_3 + $Fe(OH)_3$	0.4	95
5	CS + $CoCl_2$ + $Fe(OH)_3$	0.8	90
6	CS + CrO_3 $Fe(OH)_3$	0.8	90
7	CS + $ZnSO_4$ + $Fe(OH)_3$	0.1	98.7
8	CS + $CdCl_2$ + $Fe(OH)_3$	0.28	92
9	CS + BCl_3 + $Fe(OH)_3$	1.8	77.5
10	CS + $Pb(CH_3COO)_2^-$ + $Fe(OH)_3$	4	50
11	CS + $HgCl_2$ + $Fe(OH)_3$	ppt. formed	ppt. formed
12	CS + $CuSO_4$ + $Fe(OH)_3$	0.2	97.5

[a]The Dowex-I Cl^- fraction (1 mg/10 ml) was incubated with various trace metal ions (10 mg) and toxic metal ions (10 mg) for 1 hr at 55°C. This was followed by assay for $Fe(OH)_3$ solubilization activity. The loss in activity is represented as percent inhibition of $Fe(OH)_3$ solubilization activity of the compound. CS, compound solution.

collect the root exudates by gentle shaking in distilled deionized water for 72 hr. The Whatman No. 1 filtrate of the root exudates showed $Fe(OH)_3$ solubilization activity (Figure 1). The active $Fe(OH)_3$-solubilizing compound was purified by subjecting the Whatman No. 1 paper filtrate to Dowex I Cl^- chromatography.

TABLE 2. EFFECT OF NUTRIENT IONS ON $Fe(OH)_3$ SOLUBILIZATION ACTIVITY OF COMPOUND ISOLATED FROM *Tephrosia*[a]

S. No.	Treatment	$Fe(OH)_3$ solubilized (μg)	Percent inhibition of $Fe(OH)_3$ solubilization activity
1	CS + $Fe(OH)_3$ (control)	8	0
2	CS + $CaCl_2$ + $Fe(OH)_3$	7.25	9.3
3	CS + NaCl + $Fe(OH)_3$	8	0
4	CS + $MgSO_4$ + $Fe(OH)_3$	7.02	12.25
5	CS + KCl + $Fe(OH)_3$	7.9	1.25

[a]The Dowex-I Cl^- fraction (1 mg/10 ml) was incubated with various nutrient ions (10 mg) for 1 hr at 55°C. This was followed by normal assay for $Fe(OH)_3$ solubilization activity. The loss in activity is represented as percent inhibition of $Fe(OH)_3$ solubilization activity of the compound. CS, compound solution.

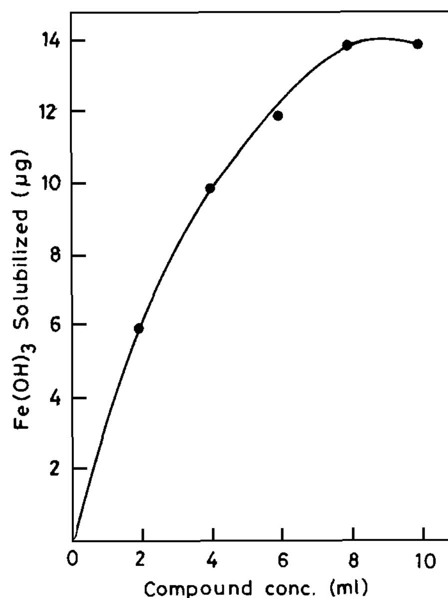

FIG. 1. Solubilization of $Fe(OH)_3$ after its treatment with the root exudate of *Tephrosia*. Fresh intact roots of *Tephrosia* were soaked in distilled, deionized water for 72 hr. The root exudates were collected and passed through Whatman No. 1 filter paper. The filtrate was tested for $Fe(OH)_3$ solubilization activity as a function of compound concentration.

The fraction that eluted between 0.1 N and 0.2 N HCl (designated as the Dowex I Cl^- fraction) showed maximum $Fe(OH)_3$ solubilization activity, and the apparent homogeneity of the fraction was evident by a single peak on HPLC (Figure 2). The purified compound retained the $Fe(OH)_3$ solubilization activity. No significant $Fe(OH)_3$ solubilization activity was observed with the Dowex I Cl^- fraction obtained from the root exudates collected in the presence of 100 μm $FeCl_3$. Consequently, the compound isolated from the root washings of *Tephrosia purpurea* functions as a phytosiderophore.

Release of the iron-solubilizing compounds and their enhanced uptake by plants growing in iron-deficient conditions have been reported by different workers. In soybean, a putative siderophore is produced in response to iron-deficiency stress (Porter, 1986); whereas nicotianamine, suggested to be phytosiderophore, has been shown to be present in a number of plant tissues (Budesinsky et al., 1980). In maize, von Wiren et al. (1994) demonstrated that the iron-inefficient mutant yellow stripe I (ysI) shows lower release of phytosiderophores and lacks an efficient uptake of phytosiderophores as compared to the wild type grown under iron-deficient in vitro cultures. Hansen et al. (1995) evaluated 17 geno-

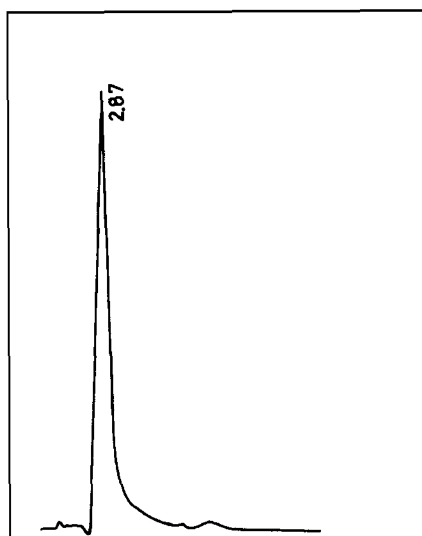

FIG. 2. Assessment of the homogeneity of $Fe(OH)_3$-solubilizing compound isolated from the root exudates of *Tephrosia*. The Dowex I Cl^- fraction that eluted between 0.1 and 0.2 N HCl was subjected to HPLC. A single peak at a retention time of 2.8 min was observed.

types of oat for the release of phytosiderophores grown under iron-limiting nutrient solutions. Their results indicate that there are differences in the amounts of phytosiderophore release by the different genotypes, and these differences are positively correlated with the field chlorosis scores. Hansen et al. (1996) also proved that the wheat cultivars resistant (Abilene) and susceptible (2157) to iron chlorosis showed differences in the release of phytosiderophores in response to foliage clipping under iron-deficient in vitro cultures. Gries and Runge (1992) screened 30 calcicolous and calcifugous grass species grown in iron-deficient nutrient solution for the release of phytosiderophores. Their observations suggest that calcicolous species release more phytosiderophores as compared to calcifugous species, and hence the ecological competitive ability of the former is higher as compared to the latter. They also correlated the low growth rates of a few calcicolous species with the release of phytosiderophores.

Tephrosia purpurea grows gregariously in pure patches on lateritic soils rich in gœthite mineral and, hence, is subjected to severe iron deficiency. However, no plant with chlorosis was observed in the natural populations. This suggests that *Tephrosia purpurea* is adapted to iron-deficiency stress by the release and uptake of phytosiderophores.

The positive response to the Bradford test for protein (Bradford, 1976) sug-

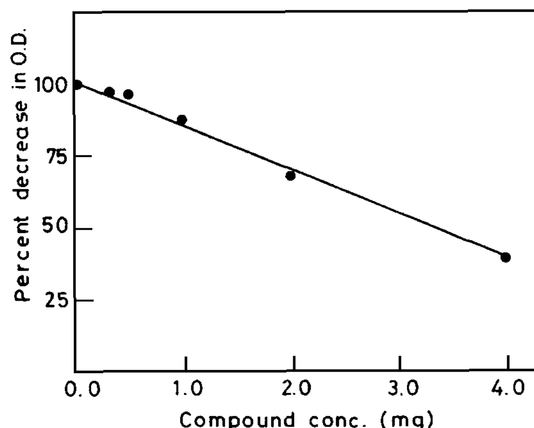

FIG. 3. Treatment of $Fe(OH)_3$-solubilizing compound with Chrome Azurol S dye. Variable concentrations of the Dowex I Cl^- fraction were incubated with Chrome Azurol S dye at 25°C for 10 min. Percent decrease in the relative absorbance at 630 nm was plotted as a function of the $Fe(OH)_3$-solubilizing compound.

gests that the Dowex I Cl^- fraction has protein linked with an iron-binding moiety. The ratio of protein content to the purified fraction was 0.0125 mg : 1.00 mg. The fact that the rabbit RBC agglutinated the fraction suggested the presence of a proteinaceous component that serves as a recognition factor. In fact, this is the first report of lectin activity of a compound that shows the properties of a siderophore. The iron-binding moiety is perhaps phenolic in nature, as the Dowex I Cl^- fraction gave a brown color with $FeCl_3$ and showed a λ_{max} at 210 and 245 nm.

The siderophore properties of the Dowex I Cl^- fraction of the root exudates are also evident from the fact that it chelated iron from the Chrome Azurol S dye (Figure 3). The Dowex I Cl^- fraction was lyophilized, and the lyophilized fraction showed $Fe(OH)_3$-solubilizing activity at the rate of 4.6 μg $Fe(OH)_3$/mg of fraction/hr (Figure 4). The iron solubilization activity of the compound was maximum at 50°C. The thermal stability indicates that the compound is active in semiarid soils where the temperature is usually in the same range (Dakshini, 1985). The enhanced activity at acidic pH (Table 3) suggests that it is active in the acidic rhizosphere of legumes. The pH in the rhizosphere of leguminous plants is usually acidic (Snowball and Robson, 1985; Young and Mytton, 1981; Date and Halliday, 1979). The effect of other trace elements and toxic ions, as listed in Table 1, on the activity of the compound was investigated. These elements and toxic ions interfered with the iron-solubilizing activity of the compound whereas cations like Na^+, K^+, Mg^{2+}, and Ca^{2+} did not (Table 2).

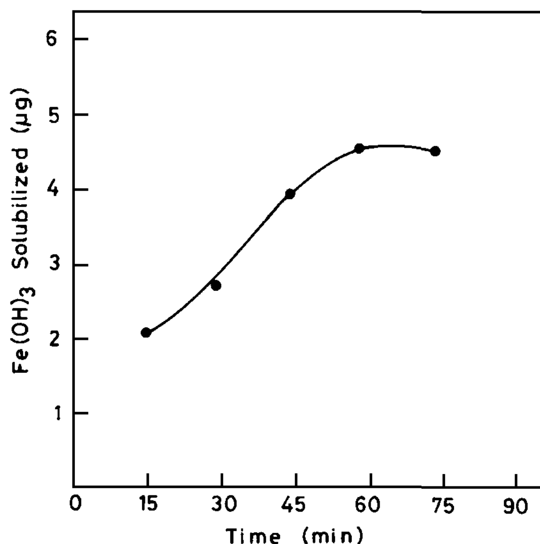

FIG. 4. The $Fe(OH)_3$ solubilization activity of the Dowex I Cl^- fraction. A $Fe(OH)_3$ solubilizing compound was purified from the root exudates of *Tephrosia* by subjecting Whatman No. 1 filter paper filtrate to Dowex I Cl^- chromatography. The Dowex I Cl^- fraction that eluted between 0.1 and 0.2 N HCl was collected and lyophilized. This Dowex I Cl^- fraction was checked for $Fe(OH)_3$ solubilization activity.

In fact, Ca^{2+} and Mg^{2+} had no inhibitory effect on iron mobilization by the phytosiderophore in barley, whereas Cu^{2+} and Zn^{2+} did (Crowley et al., 1991; Zhang et al., 1991; Treeby et al., 1989). Thus, the isolated compound from *Tephrosia purpurea* has affinity with heavy metals that compete with iron for the binding site. Consequently, the compound may serve as a remediator of heavy metals.

These results point out that some heavy metals that act as trace elements can also be mobilized by the siderophore. Our observations suggest that the compound isolated from the root exudates of *Tephrosia purpurea* not only solubilizes the $Fe(OH)_3$ but also has an affinity for other trace metals and heavy metal ions. Thus, in soils contaminated with toxic heavy metal ions that inhibit iron solubilization activity, the compound may play an important role as remediator of heavy metals.

The fact that Ca^{2+}, Na^+, and Mg^{2+} ions do not interfere with iron solubilization and chelation activity of the compound suggests that the compound may play an active role in the acquisition of iron from iron-deficient soils and may act as a bioremediator in the soils contaminated with toxic heavy metals.

TABLE 3. $Fe(OH)_3$ SOLUBILIZATION ACTIVITY OF COMPOUND AT DIFFERENT TEMPERATURES AND pH VALUES[a]

Temperature (°C)	$Fe(OH)_3$ solubilization activity [μg $Fe(OH)_3$ solubilized/mg compound]	pH	$Fe(OH)_3$ solubilization activity [μg $Fe(OH)_3$ solubilized/mg compound]
4	2	2	6
10	2	4	4
25	1.8	6	4
40	3	8	3
50	4	10	3
60	2	12	0.5

[a]The activity of the purified compound at different temperatures and pH values was determined by incubating the Dowex I Cl^- fraction (1 mg/ml) at various temperatures and pH values according to Takagi (1976).

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SHIFTS IN LIFE-HISTORY TRAITS AS A RESPONSE TO CANNIBALISM IN LARVAL LONG-TOED SALAMANDERS (*Ambystoma macrodactylum*)

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Abstract—We examined the potential influence of cannibalism on life-history characteristics of larval long-toed salamanders (*Ambystoma macrodactylum*). Using a 2 × 2 factorial design, crossing morphology with diet, we exposed typical morph larvae to one of four types of stimulus animals: cannibal morphs fed a conspecific diet, cannibal morphs fed a heterospecific diet (i.e., *Tubifex*), typical morphs fed a conspecific diet, and typical morphs fed a heterospecific diet. Test larvae exposed to stimulus animals fed a conspecific diet exhibited a slower growth rate and an increase in the time taken to reach metamorphosis. These changes in life history likely represent a cost of antipredator behavior.

Key Words—*Ambystoma macrodactylum*, long-toed salamander, cannibal morphology, cannibalism, life history traits, antipredatory behavior, chemical cues, polymorphism.

INTRODUCTION

A wide diversity of antipredator responses has evolved among prey animals. These antipredatory mechanisms can include changes in morphology (e.g., Havel and Dodson, 1984; Havel, 1987; Brönmark and Miner, 1992; McCollum and Van Buskirk, 1996; Stabell and Lwin, 1997), physiology (e.g., Wassersug, 1971;

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Smith, 1982), and behavior (e.g., Lima and Dill, 1990; Chivers et al., 1996, 1997). Behavioral responses to predators may include an increase in the use of shelter, shifts in microhabitat use, reductions in movement, and increased group cohesion (Sih, 1987; Lima and Dill, 1990; Chivers and Smith, 1998; Kats and Dill, 1998). For example, Sih (1986) found that mosquito larvae (*Culex pipiens*) reduce their activity when exposed to the insect predator *Notonecta undulata.* Alternatively, Chivers et al. (1995) found that brook stickleback increased schooling in response to the presence of predatory northern pike. Although each of these behaviors may shield an individual from predation, a trade-off commonly arises between avoiding a predator and performing other activities, including foraging (Sih, 1992). This trade-off can lead to a variety of indirect effects on long-term life-history traits of the prey.

There are many studies in which long-term effects of predators on their potential prey have been documented (e.g., Minchella and Loverde, 1981; Werner et al., 1983; Dodson and Havel, 1988; Reznick et al., 1990; Wilbur and Fauth, 1990; Skelly, 1992; Ball and Baker, 1996). Werner et al. (1983) found that the presence of predatory largemouth bass (*Micropterus salmoides*) led to a decrease in the growth rate of small bluegill sunfish (*Leopomis macrochirus*). A study of Dodson and Havel (1988) indicated that *Daphnia pulex* exposed to the nonlethal presence of the invertebrate predator, *Notonecta undulata*, exhibited a reduction in adult body size and a shorter developmental time in juveniles. Skelly (1992) showed that gray treefrog (*Hyla versicolor*) tadpoles exposed to the nonlethal presence of larval tiger salamanders (*Ambystoma tigrinum*) experienced a reduction in their growth and developmental rate. Other examples of long-term effects of predators on amphibian life history are provided by Van Buskirk (1988), Wilbur and Fauth (1990), and Skelly and Werner (1990).

Intraspecific predation (cannibalism) is common in numerous taxa, including amphibians (Fox, 1975; Polis, 1981; Smith and Reay, 1991; Crump, 1992; Elgar and Crespi, 1992; Wildy et al., 1998). Despite the widespread occurrence of cannibalism, there is little information on how intraspecific predation influences long-term life-history traits of conspecific prey. In this study we explored the possibility that cues of cannibalistic conspecifics may influence life-history characteristics of larval long-toed salamanders (*Ambystoma macrodactylum*). Larvae of this species exhibit a trophic polymorphism, whereby some individuals have a cannibalistic morphology (i.e., a disproportionately large head and hypertrophied vomerine teeth) (Walls et al., 1993a,b). Larvae lacking cannibalistic characteristics are referred to as typical morphs.

Chivers et al. (1997) documented that typical morph *A. macrodactylum* larvae responded to chemical cues but not visual cues of cannibal morphs with antipredator behavior. Specifically, typical morphs spatially avoided cannibals and showed a reduction in activity in their presence. In this experiment, we used larval *A. macrodactylum* as both predator and prey and tested whether the mor-

phology of the predator, its diet type (i.e., conspecifics or heterospecifics), or an interaction between the two, influence long-term life history characteristics of their conspecific prey. Specifically, we looked at how growth rate, time taken to reach metamorphosis, and mass at metamorphosis are influenced by intraspecific predators.

METHODS AND MATERIALS

We collected long-toed salamander larvae in July 1996 from a high-altitude (elevation 1951 m) ephemeral pond located approximately 24 km south of Sisters, Deschutes County, Oregon, USA. We transported them to the laboratory and housed individual larvae in 850-ml plastic cups containing approximately 600 ml of dechlorinated tap water. Prior to the experiment, larvae were fed *Tubifex* ad libitum every other day and were maintained on a 14L : 10D cycle.

We created experimental chambers using fiberglass mesh screen to divide glass test aquaria (50 × 25 × 30 cm) into five sections. A central area, measuring 10 × 25 cm was surrounded by four sections each measuring 12.5 × 20 cm. Within these aquaria, we raised typical morph larvae in the presence of one of four types of stimulus animals: (1) cannibal morphs fed a conspecific diet, (2) cannibal morphs fed a heterospecific diet (i.e., *Tubifex*), (3) typical morphs fed a conspecific diet, and (4) typical morphs fed a heterospecific diet. [We used the criteria of Walls et al. (1993a,b) to identify individuals with the typical and cannibal morphology]. Within each aquarium, a single typical morph test larva was placed in each of the four peripheral sections. Depending on the treatment, one of the four types of stimulus larvae was placed in the central section.

Aquaria were arranged in blocks with each block containing one of the four treatment types. This design was replicated seven times for a total of 112 animals housed in 28 aquaria. Within each block, both the stimulus animals (cannibal morphs and typical morphs) and the test animals were matched for size. The mean mass and standard error of the stimulus animals was 33.2 ± 1.3 g, while that of the test animals was 26.6 ± 2.0 g.

Throughout the experiment, test larvae were fed *Tubifex* ad libitum every other day. Stimulus animals were fed ad libitum twice per week with either *Tubifex* or larval *A. macrodactylum.* (Feeder larvae were always smaller than stimulus larvae.) Aquaria were cleaned once per week. Aquaria were initially filled to a depth of 27.5 cm. Beginning at week 6, the water level in the tanks was lowered by 2.5 cm/week to simulate natural pond drying. We began dropping the water level prior to metamorphosis of any test larvae.

We monitored the experimental aquaria daily. All test animals that reached metamorphosis (i.e., beginning of gill resorption) were removed from the test chambers and weighed. If a stimulus animal transformed prior to the end of

the experiment, we replaced it with another stimulus animal that was the same size and had the same morphology and diet. For each test animal, we calculated growth rate by dividing the difference between initial and final mass by the number of days from the beginning of the experiment to metamorphosis. For each response variable (see below), we calculated tank means for the four animals in each tank and used these means for all statistical analyses.

Our data conformed to assumptions of parametric statistics. Therefore, we used a multivariate analysis of variance (MANOVA) to examine the effects of morphology and diet of the stimulus animals on life-history traits of the test animals. After MANOVA, we used univariate analysis of variance (ANOVA) on each response variable to assess which variables were responsible for significant main effects.

RESULTS

MANOVA revealed that there was an overall effect of the treatment conditions on the life history variables that we measured (see Table 1). Subsequent ANOVAs showed that growth rate and time taken to reach metamorphosis, but not mass at metamorphosis, were influenced by the diet of the stimulus animals (Table 1, Figure 1). Neither growth rate, time taken to reach metamorphosis, nor mass at metamorphosis was influenced by the morphology of the stimulus animals (Table 1, Figure 1). There were no significant interactions between morphology and diet (Table 1, Figure 1).

DISCUSSION

Larval long-toed salamanders exhibited significant changes in life-history characteristics in response to conspecifics that had cannibalized. Larvae exhibited a significantly slower growth rate and an increased time taken to reach metamorphosis when exposed to cues from stimulus animals fed a diet of conspecifics as opposed to heterospecifics.

Prevoius studies support the idea that the diet of a potential predator can influence both short-term and long-term responses in potential prey (see reviews in Chivers and Smith, 1998; and Kats and Dill, 1998). For example, Wilson and Lefcort (1993) found that red-legged frog tadpoles reduced activity when exposed to chemical cues from newts fed a diet of red-legged frog tadpoles but not when they were fed insects. A more long-term response was demonstrated by Crowl and Covich (1990), who showed that stream snails (*Physella virgata*) exhibited faster growth and a delay in reproduction in response to cues from predatory crayfish feeding on other stream snails. In our study, we show that larval *A. macrodactlyum* experience an increased time to metamorphosis and

TABLE 1. RESULTS OF MANOVA FOR OVERALL EFFECTS OF STIMULUS TYPES (DIET AND MORPHOLOGY) ON LIFE-HISTORY TRAITS AND ANOVAS FOR EACH RESPONSE VARIABLE[a]

Source	F	P
MANOVA		
Constant	4418.57	<0.001
Diet	26.827	<0.001
Morph	1.926	0.166
Diet × morphology	0.801	0.301
ANOVAs		
Rate		
Diet	15.550	<0.001
Morphology	0.149	0.704
Diet × morphology	0.075	0.787
Time		
Diet	25.239	<0.001
Morphology	2.976	0.102
Diet × morphology	3.006	0.100
Mass		
Diet	2.024	0.172
Morphology	0.009	0.925
Diet × morphology	2.162	0.159

[a]Response variables are growth rate (rate), time taken to reach metamorphosis (time), and mass at metamorphosis (mass). Degrees of freeom are 3, 16 for MANOVAs and 1, 18 for ANOVAs.

a decreased growth rate when exposed to other larval *A. macrodactylum* that have been fed a diet of conspecifics but not when they have been fed a diet of *Tubifex*. These results are particularly interesting because predator and prey belong to the same species. In noncannibalistic populations, conspecifics may engage in competitive and/or reproductive interactions. For cannibalistic populations, predator–prey interactions may also be important.

In our study, there was no effect of the morphology of the stimulus animals on any of the life history variables that we measured. Test animals responded to the stimulus animals in the same manner, regardless of whether or not the stimulus animals had a cannibal morphology. These results differ somewhat from our past behavioral studies (Chivers et al., 1997).

Previously we found that typical morph long-toed salamander larvae exhibited antipredatory behavior in response to chemical cues of conspecific stimulus animals possessing a cannibalistic morphology. This response occurred not only when the cannibal morphs were fed a conspecific diet (as in the present study) but also when they were fed *Tubifex*. The differences in conclusions between the two experiments deserves consideration. We believe that the differences can be explained in the following way: In the behavioral experiments, the typical larvae exhibited an immediate behavioral response when presented with a predatory

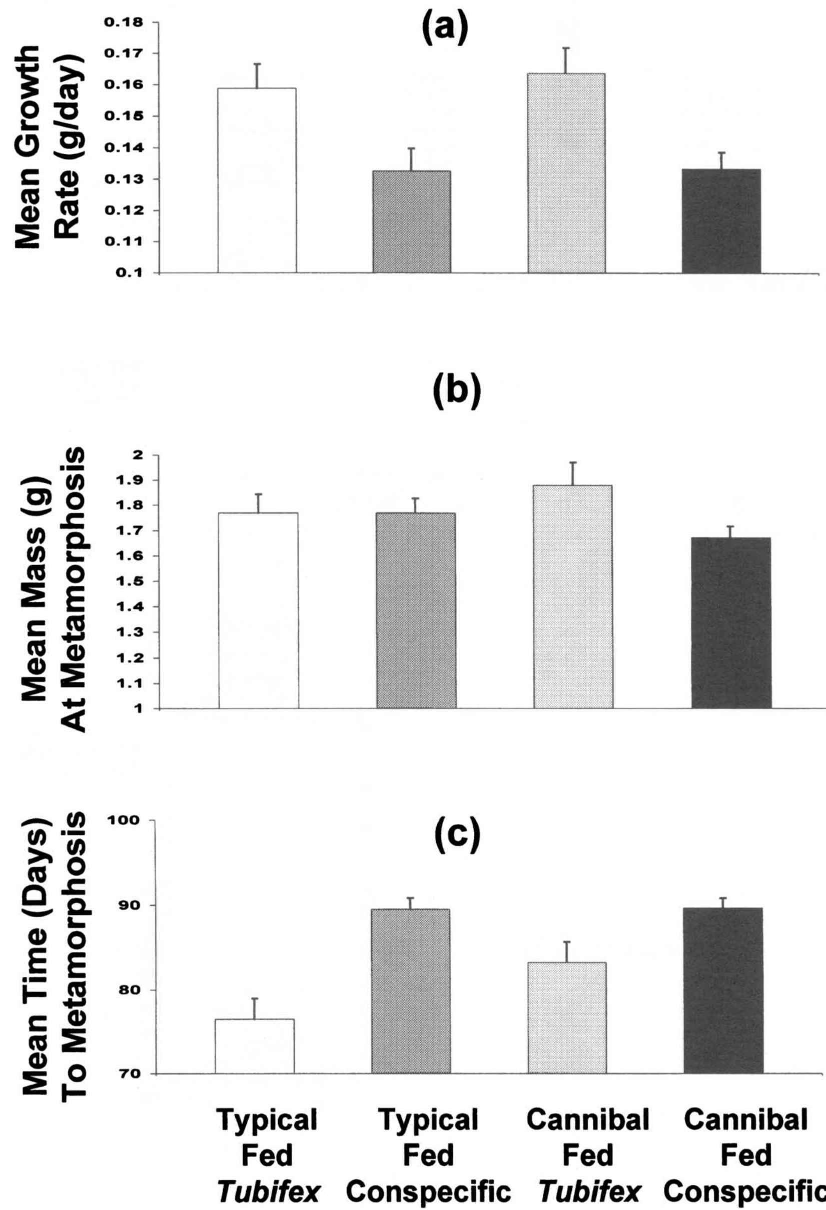

FIG. 1. (a) Mean (±SE) growth rate (grams per day) for *A. macrodactylum* test larvae, (b) Mean (±SE) mass (grams) at metamorphosis, (c) mean (±SE) time (days) to metamorphosis.

threat from a cannibal. In this instance, we only observed the behavior of the test animals for a short duration (20 min). Theory predicts that the antipredator response of the test animals to the cannibal should diminish over time if the cannibal does not attack (Lima and Dill, 1990). In the present experiment, the test animals had a long period of time to assess the potential risk posed by the cannibal. In this case, the test animals responded to the cannibals only when the cannibals acted cannibalistic (i.e., when they had consumed a conspecific diet). This interpretation is supported by examining the responses of test larvae to typical morph stimulus animals. Test animals responded to typical morph stimulus animals when the stimulus animals acted cannibalistic (i.e., had a conspecific diet), but not when the stimulus animals did not (i.e. had a *Tubifex* diet).

Long-term life-history shifts may result as by-products of antipredatory behavior (e.g., Dodson and Havel, 1988; Skelly and Werner, 1990; Skelly, 1992; Ball and Baker, 1996) or may represent facultative alterations in life history (e.g., Minchella and Loverde, 1981; Crowl and Covich, 1990; Wilbur and Fauth, 1990) or a combination of both (e.g., Skelly and Werner, 1990). We suspect that the differences in life-history traits that we observed may be a consequence or by-product of the behavioral responses of the test larvae. Chivers et al. (1997) documented that antipredator responses of larval long-toed salamanders to cues of cannibals included reduced movement and spatially avoiding the area near the cannibal. In this study, test larvae exposed to cannibals or typicals fed conspecifics may have demonstrated similar behavioral responses. These behavioral responses likely conflicted with foraging. In our experiment, all test larvae were fed ab libitum every other day. We did not attempt to determine if there were differences in the amount of food eaten by test larvae in the different treatments.

In our experiment, we observed that test animals exhibited a reduction in growth rate and a reduction in the time taken to reach metamorphosis in response to stimulus animals that were fed conspecifics over *Tubifex.* We did not observe any differences in the final mass larvae reached at metamorphosis. Other amphibians, including American toads (*Bufo americanus*), have been shown to metamorphose at a smaller size in response to the nonlethal presence of predators (Skelly and Werner, 1990).

We suggest that the responses exhibited by larvae in this study were mediated by chemical cues. Previously, we found that larval *A. macrodactylum* demonstrated antipredator behavior when exposed to both the chemical and visual cues of the cannibal morphs or chemical cues only, but not when exposed only to visual cues (Chivers et al., 1997). Moreover, the intensity of this antipredator behavior to chemical cues appeared to be elevated when the cannibal morphs were fed a diet of conspecifics (prior to behavioral trials) compared to when the cannibals were fed a diet of *Tubifex.* Therefore, we concluded that larvae were primarily using information from chemical cues to assess their sur-

roundings. Similarly, in the current study, shifts in life-history behavior demonstrated by larvae were likely a response to chemical cues arising as a by-product of the cannibal diet that the stimulus larvae had consumed.

Even though we have no evidence that larval long-toed salamanders use visual or tactile means to recognize predatory cues, we cannot completely rule out this possibility. It is possible that these cues could have been used by larvae in our experiment. The fiberglass mesh screen separating the test larvae from the stimulus larva allowed visual and tactile as well as chemical cues to be exchanged between the test and stimulus larvae. Test larvae could see the stimulus larva in the act of eating and detect any movement associated with this. Visually detecting a stimulus larva consuming a conspecific could have contributed to the shifts in life history observed.

It is not uncommon for adult long-toed salamanders to lay their eggs in temporary environments (Leonard et al., 1993; Wildy, personal observation). Consequently, it is critical for larvae developing in these habitats to metamorphose before the habitat completely dries. In our experiment, we observed that larval salamanders exhibited a reduction in growth rate and an increase in time taken to reach metamorphosis in response to the threat of cannibalism. These responses may be particularly costly in ephemeral environments; the pond may dry prior to the larvae reaching metamorphosis.

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ALLELOPATHIC POTENTIAL AND CHEMICAL CONSTITUENTS OF VOLATILE OIL FROM *Ageratum conyzoides*

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Abstract—Fresh leaves and volatile oil of the important weed *Ageratum conyzoides* in south China were highly inhibitory to seedling growth of various cultivated crops, especially in an adverse habitat. The constituents of the volatile oil were analyzed by GC-MS. Eleven components were identified and six main components, precocene I, precocene II, 3,3-dimethyl-5-*tert*-butylindone, β-caryophyllene, γ-bisabolene, and fenchyl acetate, were isolated by means of column chromatography. Precocene I, precocene II, β-caryophyllene, and 3,3-dimethyl-5-*tert*-butylindone inhibited seedling growth of acceptor plants. Inhibitory activity of the volatile oil was more intense than that of the pure components. Fenchyl acetate and γ-bisabolene have no inhibitory activity, but when mixed with precocene II, they increased the inhibitory activity to growth of acceptor seedling plants. Experiments show that allelopathic synergism exists among allelochemicals of *Ageratum conyzoides*.

Key Words—*Ageratum conyzoides*, allelopathy, volatile oil, adverse habitat, allelopathic synergism.

INTRODUCTION

Ageratum conyzoides, which originated in mid-South America, is an important and widespread weed in south China and Southeast Asia. It often becomes a dominant species in tropical and subtropical agroecosystems and invades cultivated fields, reducing crop productivity (Roder et al., 1995). It has been used for herbal medicine to cure some diseases (Tyagi et al., 1995) and as food for cattle and fish (Menut et al., 1993) in China and India. In 1976, precocene I and precocene II were isolated and identified from *A. conyzoides* and were demon-

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strated to be cytotoxic to the corpora allata, the gland that synthesizes juvenile hormone (Bowers et al., 1976). Thus, some insects treated with the precocenes precociously molt into small adults or attempt to molt too soon to the adult stage. Antibacterial activity of the essential oil from *A. conyzoides* also has been reported (Sharma et al., 1979). In our laboratory, allelopathy of *A. conyzoides* has been studied in recent years. Research showed that precocene I and precocene II were highly inhibitory to seedling growth of some cultivated crops (Hu and Kong, 1997). Further research revealed that allelochemicals of *A. conyzoides* were released through leaching and by volatilization from its aerial section, but volatilization was the primary mode (Kong et al., 1998). Because *A. conyzoides* possesses multiple biological and physiological properties, it has been intensively investigated, especially its secondary metabolites (Gonzalez et al., 1991; Wiedentold and Roeder, 1991). However, to our knowledge, the chemical constituents of the volatile oil from *A. conyzoides* have not been reported in detail (Menut et al., 1993; Riaz et al., 1995; Wandji et al., 1996).

In this paper, we analyze the chemical constituents of the volatile oil from *A. conyzoides* by means of GC-MS and determine their allelopathic potential as pure components and as mixtures on cultivated crops.

METHODS AND MATERIALS

Experimental Material. Plants of *A. conyzoides* were collected in the Ecological Experimental Station of South China Agricultural University in Guangzhou, P. R. China during August–September. Healthy and mature aerial sections were selected and divided into 1- to 2-cm pieces. The volatile oils were obtained immediately by means of steam distillation from fresh material after collection. Uniform seeds of cucumber (*Cucumis sativus* L.), ryegrass (*Lolium ultiforum* L.), radish (*Raphanus sativus* L.), mungbean (*Phaseolus aureus*), wheat (*Triticum aestivum* L.), and tomato (*Lycapesicon*) were procured from the Agronomy Department of South China Agricultural University. Silica gel (200–300 mesh) and organic solvents were of analytical grade purity and were commercial products from Chinese markets.

Bioassay of Volatile Oil and Main Allelochemicals. The bottom halves of 10-cm-diam. Petri dishes were covered with Whatman No. 40 filter paper disks laid on a thin layer of quartz sand. The paper and sand were moistened with 2.5 ml Hoagland's nutrient solution (Hoagland and Arnon, 1950). Thirty uniform, germinated seeds [radicle lengths about 1 mm, programmed according to the rules of the International Seed Testing Association (1976)] of each acceptor plant species were placed on a Petri dish (five dishes each treatment). The uncovered Petri dishes were placed in an environmentally controlled chamber (3 m^3), in which there was a source of fresh leaves, the volatile oil, or selected allelochemicals from *A. conyzoides*. Volatility of chemical components was maintained with

proper air displacement pressure and monitored by gas chromatography (GC). Ordinary air in the chamber served as the control. Acceptor plants were grown for a week under a cycle of 12L : 12D photoperiod (236 $\mu E/sec/m^2$) at 28°C and 23°C, respectively. The shoot height, root length, and chlorophyll content of 50 seedlings comprised of 10 seedlings from each of five dishes were measured. Determination and calculation of chlorophyll content were based on the methods of Baziramakenga et al. (1994) and Knudson et al. (1977). All experiments were conducted under the same conditions and were repeated three times, with each time representing one replicate in the statistical analysis.

Analysis of Volatile Oil. Components of the volatile oil were separated on an HP 5890 gas chromatograph equipped with a flame ionization detection (FID). Samples were injected in the splitless mode (1 μl) in an HP-1 with a capillary column (50 m × 0.32 mm bonded-phase fused silica column). The initial oven temperature was maintained at 60°C for 2 min and then programmed at 5°C/min to 280°C. Helium was the carrier gas with a head pressure of 12.0 psi.

GC-MS analyses were performed on a Finnigan TSQ70B GC-MS-MS model 4000 instrument. The same chromatographic conditions and capillary column as described above were used. Ionization was determined in the election impact mode (EI) at 50 eV, 280°C. The mass spectrum was repetitively scanned from 35 to 450 amu every 2 sec. Data acquisition and analysis were accomplished with a Finnigan-Incos data system. The chemical constituents of the volatile oil were identified by a peak matching library search, published standard mass spectra, and by analysis of authentic reference compounds (Aldrich Chemical Co.).

Isolation and Identification of Main Allelochemicals. A portion of the total oil (5 g) was placed on a 40- × 60-cm silica gel column and eluted with hexane, hexane–ethyl acetate (10 : 1 v/v), and ethyl acetate to give three fractions. Solvents were removed by a rotary evaporator. γ-Bisabolene (280 mg) and β-caryophyllene (510 mg) were isolated from fractions eluted with hexane. Precocene I (1.1 g), precocene II (1.7 g), and fenchyl acetate (390 mg) were isolated from the fraction eluted with hexane–ethyl acetate, and 3,3-dimethyl-5-*tert*-bulylindone (430 mg) was isolated from the fraction eluted with ethyl acetate. All isolated allelochemicals were identified by authentic reference compounds. 3,3-Dimethyl-5-*tert*-butylindenone was further identified by means of IR (Shimadzu IR-435 mode) and ^{1}H NMR (Brucker AM 400 mode).

Analysis of Data. Statistical comparisons of treatments were made by the method of Williamson and Richardson (1988). The response index (RI) was determined as follows:

$$\text{If } T \geq C, \text{then } RI = 1 - C/T$$
$$\text{If } T < C, \text{then } RI = T/C - 1$$

where T is the treatment response and C is the control response. RI values range from +1 to −1, with positive values indicating stimulation by the treatments and negative values indicating inhibition relative to the controls. Absolute values of RI varied directly according to allelopathic effects.

RESULTS

Allelopathic Potential of Fresh Leaves and Volatile Oil. The allelopathic effect of fresh leaves and the volatile oil from *A. conyzoides* on seedling growth of various cultivated crops were determined. The results showed that both fresh leaves and the volatile oil were highly inhibitory to acceptor plants (Table 1). Inhibition of shoot height was stronger than that of root length. The roots of the acceptor plants were not able to directly contact the volatile allelochemicals under the experimental conditions. Overall, the allelopathic effects of the volatile oil were greater than that of the fresh leaves.

Chemical Constituents of Volatile Oil. The structures of 11 compounds in the volatile oil from *A. conyzoides*, analyzed by means of GC-MS, were identified (Figure 1). Six main compounds, which comprised more than 95% of the volatile oil, were isolated by column chromatography. They were precocene I, precocene II, 3,3-dimethyl-5-*tert*-butylindone, β-caryophyllene, γ-bisabolene, and fenchyl acetate. The volatile oil content of the plant and its components varied with different habitats. However, precocene I, precocene II, 3,3-dimethyl-5-*tert*-butylindone, β-caryophyllene, γ-bisabolene, and fenchyl acetate were always main components.

Three different habitats were selected in the Ecological Experimental Sta-

TABLE 1. ALLELOPATHIC EFFECTS OF VOLATILE OIL AND FRESH LEAVES (IN PARENTHESES) ON SEEDLING GROWTH OF CROPS FROM *A. conyzoides*

	RI_a[a]					
Item	Cucumber	Wheat	Radish	Ryegrass	Tomato	Mungbeam
Fresh weight	−0.29b	−0.19a	−0.34a	−0.26a	−0.32b	−0.16a
	(−0.24a)	(−0.13a)	(−0.29a)	(−0.11a)	(−0.29b)	(−0.10a)
Root length	−0.48b	−0.13a	−0.39a	−0.39b	−0.38b	−0.18a
	(−0.41a)	(−0.12a)	(−0.33a)	(−0.30b)	(−0.21b)	(−0.15a)
Shoot height	−0.55b	−0.37b	−0.50a	−0.49a	−0.59b	−0.25b
	(−0.49b)	(−0.31b)	(−0.51a)	(−0.44a)	(−0.55b)	(−0.21b)

[a]RI_a: mean of response index of allelopathic effects in same treatment with three replicates. A t test was used to evaluate the difference between treatment and control. A significant difference at α = 0.05 is indicated by the letter "a" following a number, and significance at α = 0.001 is indicated by the letters "b."

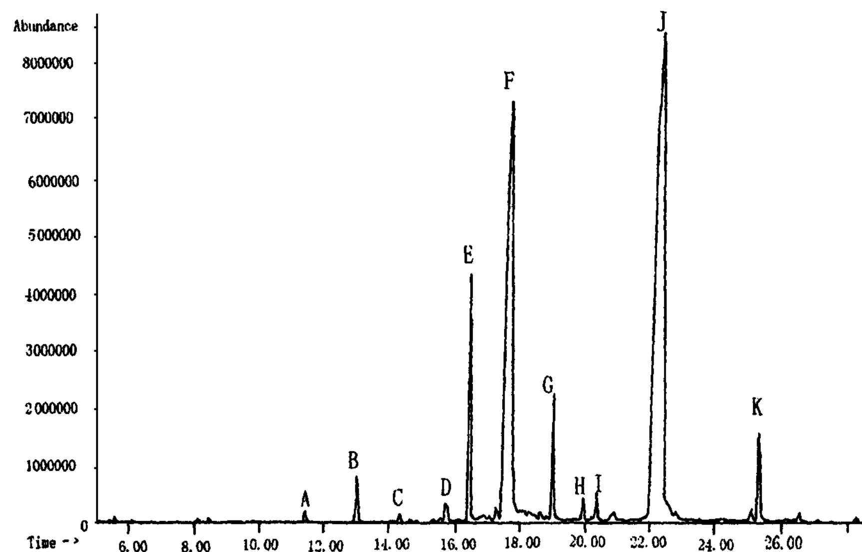

FIG. 1. The GC-MS analytical results of the volatile oil from *A. conyzoides*. A: Bornyl acetate (*endo*-bicyclo[2,2,1]heptan-2-ol-1,7,7-trimethyl acetate), B: fenchyl acetate (*endo*-bicyclo[2,2,1]heptan-2-ol-1,3,3-trimethyl acetate, C: nerol, D: α-cublene, E: β-caryophyllene, F: precocene I, G: γ-bisabolene, H: *cis*-nerolidol, I: β-caryophyllene oxide, J: precocene II, K: 3,3-dimethyl-5-*tert*-butylindone, [5-(1,1-dimethylethyl)-2,3-dihydro-3,3-methyl-1H-inden-1-one]. IR (film), 1709.6 cm^{-1} (C=O). ^1H NMR($CDCl_3$), 1.36 (*gem*-CH_3); 1.44 (t-Bu), 2.49 (CH_2), 0.32–7.51 (ArH) ppm.

tion of South China Agricultural University for study of the effect of habitat on the volatile oil and its components in *A. conyzoides* (Table 2). Sampling points A and C produced healthier *A. conyzoides* and the fresh weight of a single plant was at least 350 g. Sampling point B was dry and had low organic material and nutrients. This habitat represented poor growing conditions for *A. conyzoides*; the biomass of *A. conyzoides* in habitat B was low, and the average fresh weight was about 150 g per plant. However, *A. conyzoides* from habitat B had stronger allelopathic potential and the precocene II content of plants increased. The experiments showed that healthier *A. conyzoides* produce less allelochemicals and have weak allelopathic potential. Resource stress induced by drought and under nutrient-limited environments are known to enhance the production of secondary metabolites, which could result in intensification of allelopathic effects.

Allelopathic Effects of Volatile Oil and Its Main Components. The allelopathic effects of the volatile oil of *A. conyzoides* and of its main components are listed in Table 3. The results show that precocene I, precocene II, β-caryophyl-

TABLE 2. ALLELOPATHIC EFFECTS AND PRECOCENE II CONTENT OF *A. conyzoides* FROM DIFFERENT HABITATS

	Soil condition								Allelopathic effects (RI_a)[b]	
	Water content									
Sampling point	%	pH	Organic matter (%)	Avail. N (μg/g)	Avail. P (μg/g)	Avail. K (μg/g)	Sunlight condition	Precocene II[a] (μg/g)	Root length	Shoot height
A	17.11	6.19	6.14	222.4	37.2	198.4	Semishaded by trees	997.5	−0.26b	−0.35b
B	6.30	6.49	1.50	54.2	6.0	126.7	No trees	3973.9	−0.59b	−0.68b
C	10.07	6.94	4.01	116.0	15.9	139.6	Shaded by trees	734.6	−0.21a	−0.32b

[a]Determined by Gilson HPLC, Whatman 110 ODS-3 (22 × 500 mm) prepared column, Gilson model 116 detector. Mobile phase: CH_3OH/H_2O.
[b]Action of fresh leaves on wheat as the acceptor plant. "a" and "b" are as defined in Table 1 footnote.

TABLE 3. ALLELOPATHIC EFFECTS OF VOLATILE OIL AND ITS MAIN COMPONENTS OF *A. conyzoides*

Allelo- Chemicals	Volatility* (mg/hr)[a]	Root length (RI_a)[b]			Shoot height (RI_a)[b]			Chlorophyll of Radish[b]	
		Radish	Mungbean	Tomato	Radish	Mungbean	Tomato	*a*	*a* + *b*
Volatile oil	201	−0.64b	−0.43b	−0.55b	−0.66a	−0.78b	−0.58b	−0.42b	−0.34b
Precocene I	130	−0.53a	−0.41a	−0.43a	−0.40a	−0.61b	−0.46b	−0.32a	−0.26b
Precocene II	13	−0.32b	−0.24b	−0.42a	−0.31a	−0.47a	−0.37a	−0.21a	−0.17a
β-Caryophyllene	110	−0.43b	−0.32b	−0.50a	−0.48a	−0.64b	−0.50a	−0.32a	−0.25a
3,3-Dimethyl-5-*tert*-butylindone	59	−0.39b	−0.11a	−0.12a	−0.25a	−0.24a	−0.21a	−0.21a	−0.13a
γ-Bisabolene	52	−0.07	−0.05a	−0.03	−0.05	−0.09a	−0.04	0.01	0.03
Fenchyl acetate	53	−0.03	0.03	−0.02	0.04	−0.06	0.03	0.03	−0.03

[a]Volatility was maintained with the proper air displacement pressure and detected by GC under study.
[b]"a" and "b" are as defined in Table 1 footnote.

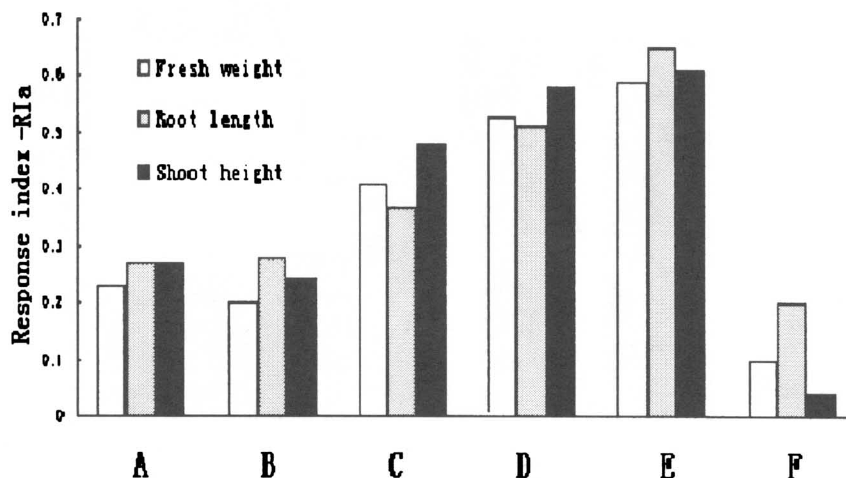

FIG. 2. Allelopathic synergism among allelochemicals of *Ageratum conyzoides*. A: precocene II, B: precocene II + precocene I, C: precocene II + γ-bisabolene, D: precocene II + fenchyl acetate, E: precocene II + γ-bisabolene + fenchyl acetate, F: γ-bisabolene + fenchyl acetate.

lene, and 3,3-dimethyl-5-*tert*-butylindone are inhibitory to seedling growth of radish, mungbean, and tomato. The chlorophyll content of radish seedling was decreased. However, γ-bisabolene and fenchyl acetate did not inhibit seedling growth of acceptor plants or decrease chlorophyll content of radish seedling. Each component of the volatile oil of *A. conyzoides* played a different role in phytotoxicity. The inhibitory effects on acceptor plants of the volatile oil were more intense than the effects of its individual components. The more volatile the compound, the stronger its inhibitory effect, except for precocene II, which was a solid and less volatile. Nevertheless, its inhibitory effects on acceptor plants were stronger. Mixtures of precocene I and precocene II did not enhance the allelopathic effects on acceptor plants (radish). However, the allelopathic effects of a mixture of precocene II and γ-bisabolene or fenchyl acetate on radish were intensified (Figure 2), although γ-bisabolene and fenchyl acetate were not highly inhibitory to seedling growth of the acceptor plants. Thus, relatively inactive allelochemicals in *A. conyzoides* have a synergistic effect in combination with precocene II.

DISCUSSION

Precocenes, the main components of the volatile oil of *A. conyzoides* allatectomize some insects (Bowers et al., 1976) and possess antibacterial activ-

ity (Sharma et al., 1979). In this paper, we have shown that precocenes and the volatile oil of *A. conyzoides* inhibited seedling growth of various cultivated crops, and the vapors of the volatile oil and its components lowered the chlorophyll content of the receiving plant. Through volatilization, *A. conyzoides* releases allelochemicals that affect neighbor plant growth and defend against attack of insects and bacteria. Thus, *A. conyzoides* has the potential to become an important species in agroecosystems. The biological activity of precocenes against some organisms in the agroecosystem suggest potential for development of novel control strategies.

The constituents of the volatile oil from *A. conyzoides* are terpenoid compounds. No nitrogen-containing components were identified. Each component of the volatile oil from *A. conyzoides* had a different inhibitory impact on acceptor plants. Various combinations of precocene II and other components acted synergistically to inhibit growth of acceptor plants, illustrating the principle that it is not always necessary that a single inhibitory compound be present in large quantity in order to affect growth of a receiving plant (Einhellig, 1996). Allelochemicals released from a plant often include compounds of different chemical categories. Thus, differences in the inhibitory action of each component on a receiving plant are likely, and antagonistic, additive, or synergistic actions among components are possible.

The allelopathic potential of *A. conyzoides* was different in different habitats. When *A. conyzoides* grew in an adverse habitat, it showed stronger allelopathic potential, and the content and constituents of its volatile oil varied with habitat. The allelopathic effects of *A. conyzoides* were strongly coupled with stresses in its environment. Thus, further research on the chemical ecology of *A. conyzoides* under environmental stress, including insect and disease attack, and under different climate and nutrient variables and their interactions are warranted.

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SEX-RELATED PERCEPTION OF INSECT AND PLANT VOLATILES IN *Lygocoris pabulinus*

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Abstract—We recorded electroantennograms of male and female *Lygocoris pabulinus* antennae to 63 insect and plant volatiles. EAGs were between 100 and 500 μV. Overall, male EAGs were about twice the size of female EAGs. In both sexes, largest EAGs were recorded to (*E*)-2-hexenyl butanoate and (*E*)-2-hexen-1-ol. Response profiles were similar in both sexes. However, male antennae were more sensitive to a number of esters, especially the butanoates and pentanoates. Female antennae were more sensitive to nine of the 19 plant volatiles, i.e., to hexan-1-ol, heptan-1-ol, 1-octen-3-ol, 2-heptanone, (*R*)-carvone, linalool, geraniol, nerol, and methyl salicylate. Sexual differences in responses suggest that males are more sensitive to insect-produced pheromone-type compounds, whereas females are more sensitive to plant compounds for their orientation towards oviposition sites.

Key Words—Heteroptera, Miridae, green capsid bug, sex pheromone, electroantennogram, odors, plant volatiles, esters, (*E*)-2-hexenyl butanoate, (*E*)-2-hexen-1-ol.

INTRODUCTION

Female-produced sex pheromones are present in at least 10 mirid bug species (Strong et al., 1970; King, 1973; Smith, 1977; Boivin and Steward, 1982;

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Graham, 1987; Graham et al., 1987; Smith et al., 1991, 1994; Chinta et al., 1994; Millar et al., 1997; Millar and Rice, 1998). However, identification of the components and their active ratio has been elucidated in three species only (Smith et al., 1991; Millar et al., 1997; Millar and Rice, 1998). The pheromones of these species have been identified by analyzing chemical differences between male and female extracts. A sex pheromone from the green capsid bug *Lygocoris pabulinus* (L.) (Heteroptera: Miridae) is active in the field, where males are attracted to traps baited with virgin live females caged on a potato shoot (Blommers et al., 1988). The pheromone of this species is needed for development of integrated pest management in fruit orchards in northwestern Europe where *L. pabulinus* is a serious pest (Blommers, 1994; Ravn and Rasmussen, 1996). Pheromone traps are widely used for monitoring lepidopterous pests (e.g., Minks and Van Deventer, 1992), and since 1990, also for monitoring the mirid pest *Campylomma verbasci* (McBrien et al., 1994). In our quest to identify the sex pheromone of *L. pabulinus*, consistent chemical differences between the sexes have not yet been found (unpublished data). In order to screen for potential attractants, perception of a number of volatiles was studied by means of electroantennogram (EAG) recordings.

The EAG technique is regularly used as a bioassay to test olfactory perception in insects (e.g., Dickens et al., 1993a,b; Visser and Piron, 1995; Visser et al., 1996). In mirids, the technique has been applied to *Lygus lineolaris* (Chinta et al., 1994; Dickens et al., 1995), revealing olfactory receptors on the antennae that are responsive to insect and plant volatiles.

Two groups of volatiles were chosen for testing. The first consisted of esters that have been identified in other bug species (Knight et al., 1984; Graham, 1988; Smith et al., 1991; Chinta et al., 1994; Aldrich, 1995; Dickens et al., 1995; Millar et al., 1997; Millar and Rice, 1998). The second consisted of general plant volatiles, of which some may play a synergistic role in the sexual attraction of *L. pabulinus* (Groot et al., 1996). EAGs of both males and females were recorded because volatiles eliciting larger EAGs in males could indicate a role in sexual attraction.

METHODS AND MATERIALS

Insects. *L. pabulinus* was reared under summer conditions (22 ± 2°C, 65 ± 5% relative humidity, 18L : 6D) on potted potato plants (Blommers et al., 1997). Males and females were separated 0–2 days after the final molt to adult. *L. pabulinus* becomes sexually active about four days after the final molt (Groot et al., 1998a), and so for EAG recordings, sexually mature, virgin adults 4–10 days old were used. Prior to EAG recordings, bugs were collected from a rearing cage containing potato plants and sexed conspecifics.

Chemicals. Olfactory stimuli used are listed in Table 1. A series of esters was tested, comprising acetates, propionates, butanoates, pentanoates, and hexanoates. A series of plant-related volatiles were also tested, comprising one arbitrarily chosen nitrile (4-methoxy phenylacetonitrile), methyl salicylate, six monoterpenes, three ketones, five alcohols, and one aldehyde. (*E*)-2-Hexenyl acetate and (*Z*)-3-hexenyl acetate were tested in both series, since these compounds are insect-produced esters as well as general green leaf volatiles (see references in Table 1). Chemicals were obtained from commercial sources or were synthesized (Table 1). Newly synthesized esters [except (*E*)-2-butenyl and (*E*)-2-octenyl esters] were prepared by refluxing a twofold excess of the appropriate carboxylic acid with the corresponding alcohol for 5 hr, followed by base extraction and partition into diethyl ether with removal of the solvent in vacuo. The (*E*)-2-butenyl and (*E*)-2-octenyl esters were prepared by refluxing 1 equivalent (eq) of the acid chloride with 1 eq of the corresponding alcohol for 1 hr, following Vogel (1989). (*E*)-2-Butenyl alcohol contained ~5% of the *Z* isomer; (*E*)-2-octenyl alcohol was prepared by reducing (*E*)-2-octenal with $NaBH_4$. Purity was determined by GC-MS. The (*E*)-2-butenyl esters contained ~5% of the *Z*-isomer, which was in correspondence with the 5% *Z* isomer in the starting material. For the EAG recordings, all chemicals were dissolved in paraffin oil at 1% v/v, following Visser and Piron (1995).

Antennal Preparation. An individual bug was anesthetized with CO_2 for a few seconds. The head was clipped off, and the distal tip of the terminal segment of one antenna cut off. The ground electrode was inserted into the open side of the head, and the recording electrode was sleeved over the tip of the antenna. The electrodes consisted of two glass capillaries filled with 0.1 M KCl solution. *L. pabulinus* antennae prepared this way showed a life-span of 15–30 min.

EAG Protocol. Ag–AgCl wires in the glass electrodes connected the antennal preparation to the amplification and recording devices consisting of an input probe and DC amplifier (Grass HIP 16A and P16D, rise time set at 30 msec), an oscilloscope (Philips PM3302), and a transient recorder (Krenz TRC 4010, 12 bits ADC) connected to a personal computer (Estate 80386 and 80387). Stimulation cartridges were prepared by applying 25 μl of each paraffin oil solution onto a piece of filter paper that was subsequently placed in a Pasteur pipet. The antenna, placed perpendicularly 1–2 cm in front of a glass tube, was stimulated for 2 sec by pushing air (1 ml/sec) through the pipet into the tube with a continuous airflow of 40 cm/sec (30 ml/sec). The interstimulus time interval was 30 sec. In order to compare responses within an individual and among individuals, all responses were normalized by using a standard of 1% (*E*)-2-hexenal in paraffin oil. The stimulation of each chemical was, thus, preceded and followed by the standard.

All chemicals were tested with 11–14 different individuals of each sex. Chemicals were tested in series of 10–18 compounds per antenna, each time

TABLE 1. VOLATILES USED FOR EAG RECORDINGS OF *L. pabulinus* MALES AND FEMALES

Volatile	Source	Purity (%)	Referred to as insect-produced odor[a]	Referred to as plant-produced odor[a]
Esters				
Butyl acetate	Acros[b]	>99	5	
(*E*)-2-Butenyl acetate	[c]	93		
Pentyl acetate	Roth[d]	>99		
Hexyl acetate[e]	Fluka[f]	99	1, 2a, 3a, 4, 5, 6	22
(*E*)-2-Hexenyl acetate	ICN/K&K[g]	99	2, 3, 4, 6, 7	23, 24, 25
(*Z*)-3-Hexenyl acetate	Roth	99	2, 7	19, 20, 21, 22, 24, 25, 26
Heptyl acetate	Roth	>99		
Octyl acetate	Roth	>99	2, 3, 5	
(*E*)-2-Octenyl acetate	[c]	62[h]	2, 3a, 4, 5, 7, 8	
Butyl propionate	Acros	>99		
(*E*)-2-Butenyl propionate	[c]	93		
Pentyl propionate	[c]	97		
Hexyl propionate	Roth	>99		19
(*E*)-2-Hexenyl propionate	ICN/K&K	98		25
(*Z*)-3-Hexenyl propionate	ICN/K&K	>99		25
Heptyl propionate	[c]	96		
Octyl propionate	[c]	96		
(*E*)-2-Octenyl propionate	[c]	79[i]		
Butyl butanoate	Roth	>99	1a, 4, 5, 6	
(*E*)-2-Butenyl butanoate	[c]	93	1a	
Pentyl butanoate	Roth	>99		
Hexyl butanoate[e]	Roth	>99	1, 2, 3, 4, 5, 6, 9	23
(*E*)-2-Hexenyl butanoate[e]	[c]	85[i]	6, 10	
(*Z*)-3-Hexenyl butanoate	[c]	92		19, 21, 20
Heptyl butanoate	[c]	97		
Octyl butanoate	Roth	>99		

(*E*)-2-Octenyl butanoate	*c*	92	2a, 3	
Butyl pentanoate	*c*	95		
(*E*)-2-Butenyl pentanoate	*c*	95		
Pentyl pentanoate	*c*	96		
Hexyl pentanoate	*c*	95		
(*E*)-2-Hexenyl pentanoate	*c*	86[i]		
(*Z*)-3-Hexenyl pentanoate	*c*	97		
Heptyl pentanoate	*c*	93		
Octyl pentanoate	*c*	93		
(*E*)-2-Octenyl pentanoate	*c*	88[i]		
Butyl hexanoate	Roth	>99	4	23
(*E*)-2-Butenyl hexanoate	*c*	95		
Pentyl hexanoate	*c*	97		
Hexyl hexanoate	*c*	97	5	
(*E*)-2-Hexenyl hexanoate	*c*	92	5, 11	
(*Z*)-3-Hexenyl hexanoate	*c*	84[i]		
Heptyl hexanoate	*c*	97		
Octyl hexanoate	*c*	97		
(*E*)-2-octenyl hexanoate	*c*	92		
Aldehydes				
Hexanal	Fluka	98	4, 7	19, 25, 26
(*E*)-2-Hexenal (standard)	Roth	96	4, 6, 7, 11, 12, 13, 14, 15, 16	6, 19, 22, 24, 25, 26, 27
Alcohols				
Hexan-1-ol[e]	Fluka	99	1, 3, 4, 7	10, 19, 21, 22, 25, 26, 27
(*E*)-2-Hexen-1-ol	Roth	97	7	19, 22, 24, 25, 26, 27
(*Z*)-3-Hexen-1-ol	Roth	97		6, 19, 20, 22, 25, 26, 27
Heptan-1-ol	Fluka	99		6, 22, 25
1-Octen-3-ol	Fluka	98		(1), 21, 25
Ketones				
2-Heptanone	Aldrich[j]	98	8, 12	25, 26
3-Heptanone	Aldrich	98		25
3-Octanone	Aldrich	99		25

TABLE 1. CONTINUED

Volatile	Source	Purity (%)	Referred to as insect-produced odor[a]	Referred to as plant-produced odor[a]
Monoterpenes				
(1S)-β-Pinene	Fluka	99	7, 17	19, 25, 26
Myrcene	Roth	91	17	20, 22, 25
(*R*)-Carvone	Aldrich	98		25, 26
Linalool	Fluka	97	7, 16, 18	6, 21, 22, 20, 25, 26
Geraniol	Fluka	99		6, 22, 25, 26
Nerol	Aldrich	97		6, 25, 26
Aromatics				
Methyl salicylate	Fluka	99		20, 21, 24
4-Methoxylphenylacetonitrile	Fluka	97		28

[a]Numbers in the list refer to the following references: **1.** Smith *et al.* (1991) (**1a:** sex pheromone compound *Campylomma verbasci*; **1:** from mold growing on mullein); **2.** Millar et al. (1997) (**2a:** sex pheromone compound *Phytocoris relativus*); **3.** Millar and Rice (1998) (**3a:** sex pheromone compound *Phytocoris californicus*); **4.** Aldrich and Yonke (1975); **5.** Knight et al. (1984); **6.** Chinta et al. (1994); **7.** Aldrich (1995); **8.** Blum (1985): **9.** Graham (1988); **10.** Dickens et al. (1995); **11.** Leal and Kadosawa (1992); **12.** Blum (1996); **13.** Ishiwatari (1974); **14.** Ishiwatari (1976); **15.** Borges and Aldrich (1992); **16.** Aldrich (1988); **17.** Staddon (1990); **18.** Aldrich et al. (1993); **19.** Bernays and Chapman (1994); **20.** Bolter et al (1997); **21.** Dicke et al. (1990); **22.** Dickens et al. (1993b); **23.** Fein et al. (1982); **24.** Takabayashi et al. (1994); **25.** Visser and Piron (1995); **26.** Visser et al. (1996); **27.** Visser et al. (1979); **28.** Cole (1976).

[b]Geel, Belgium.

[c]Synthesized de novo.

[d]Karlsruhe, Germany.

[e]Compound found in both sexes of *L. pabulinus* (F.P. Drijfhout, unpublished research).

[f]Buchs, Switzerland.

[g]Costa Mesa, California.

[h]About ~32% of the rest product is 1-octenyl acetate, the remaining 5% consists of the alcohol and the acid from which (*E*)-2-octenyl acetate was synthesized.

[i]The remaining percentage consists of the starting compounds, i.e., the alcohol and the acid, from which the listed compounds were synthesized.

[j]Zwijndrecht, The Netherlands.

offered in a different order. As a control, an EAG to 25 μl of paraffin oil was recorded during each series. EAGs of all chemicals were expressed as percentage responses relative to the responses of the adjacent standards. If the magnitude of the EAG of the adjacent standard was below 100 μV, the EAG was omitted. Means and 95% confidence intervals were calculated for each compound. Differences in volatility among compounds were not corrected for.

Statistical Analysis. The series of 45 esters was analyzed separately from the series of plant compounds. (*E*)-2-Hexenyl acetate and (*Z*)-3-hexenyl acetate were tested and analyzed in both series. Both series were analyzed by using a mixed linear model, fitted with the procedure MIXED of the computer program SAS (1997) version 6.12. Data were square-root transformed after addition of a constant in order to normalize and stabilize the variance. After fitting the mixed linear model with fixed main effects and interaction for gender and chemicals and a random effect for the antenna, the following comparisons were made:

A1: Response to the control paraffin oil versus response to each of the tested esters in female antennae.
A2: Response to the control paraffin oil versus response to each of the tested esters in male antennae.
A3: Female versus male antennal response to each of the tested esters.
B1: Response to the control paraffin oil versus response to each of the tested plant compounds in female antennae.
B2: Response to the control paraffin oil versus response to each of the tested plant compounds in male antennae.
B3: Female versus male antennal response to each of the tested plant compounds.

The significance level of each series was corrected for multiple comparisons through the Bonferroni method, and, therefore, set at: for A1 and A2, $P = 0.05/45 = 0.0011$; for A3, $P = 0.05/46 = 0.0011$; for B1 and B2, $P = 0.05/20 = 0.0025$, and for B3, $P = 0.05/22 = 0.0023$.

RESULTS

The absolute peak EAG response of *L. pabulinus* to the standard (*E*)-2-hexenal was larger in males than in females; the male response was −308 ± 142 μV (mean ± SD; $N = 9$), the female response was −149 ± 51 μV (mean ± SD; $N = 9$).

Esters (Figure 1, Table 2)

A1. Responses in Female Antennae. Female antennae were most responsive to (*E*)-2-hexenyl butanoate (166 ± 13% (mean ± CI) relative to the stan-

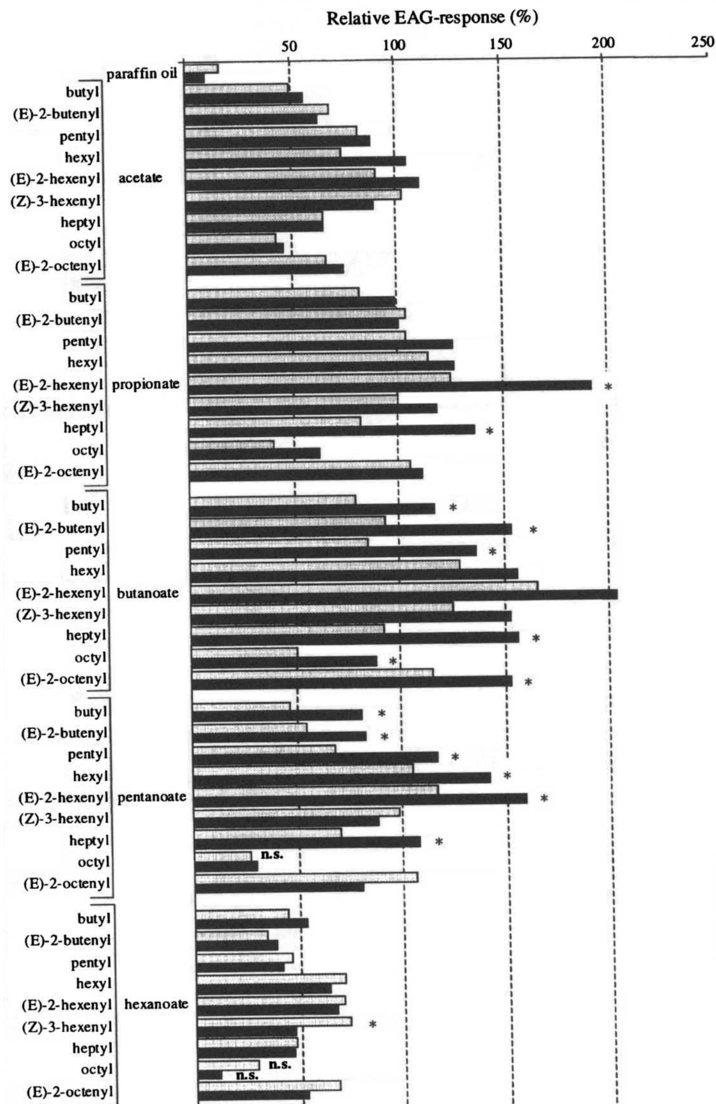

FIG. 1. EAG responses of male and female *L. pabulinus* to the series of esters. The response bars are based on the back-transformed least-square means. Grey: EAG response of female antennae; black: EAG response of male antennae; n.s.: EAG response not significantly different from the response to the control paraffin oil. *EAG response significantly different between the sexes (after correcting the significance level for multiple comparisons through the Bonferroni method). See text for further explanation.

TABLE 2. ANOVA TABLE OF SERIES OF ESTERS (RESPONSE PROFILES ARE SHOWN IN FIGURE 1)

Tests of fixed effects, source	NDF[a]	DDF[b]	Type III F	Pr > F	Covariance parameter	Estimate
Gender	1	122	27.28	0.0001	Antenna (gender)	0.5603
Chemical	45	1002	146.98	0.0001	Residual	0.9225
Gender × chemical	45	1002	8.47	0.0001		

[a]Numerator degrees of freedom.
[b]Denominator degrees of freedom.

dard). Slightly lower responses were elicited by hexyl propionate, (*E*)-2-hexenyl propionate, hexyl butanoate, (*E*)-3-hexenyl butanoate, (*E*)-2-octenyl butanoate, hexyl pentanoate, (*E*)-2-hexenyl pentanoate, and (*E*)-2-octenyl pentanoate. Lowest responses were recorded for the octyl esters and hexanoates, as in the male antennae. Octyl pentanoate and octyl hexanoate did not elicit a significant EAG response in female antennae.

A2. Responses in Male Antennae. The EAG profile of the males showed a distinct sensitivity for the array of esters. Largest EAGs were elicited by (*E*)-2-hexenyl propionate (195 ± 15% mean ± CI), and (*E*)-2-hexenyl butanoate (204 ± 15% mean ± CI). (*E*)-2-Butenyl butanoate, hexyl butanoate, (*Z*)-3-hexenyl butanoate, heptyl butanoate, (*E*)-2-octenyl butanoate, hexyl pentanoate, and (*E*)-2-hexenyl pentanoate elicited EAGs around 150% relative to the standard in male *L. pabulinus* antennae. The smallest EAGs were recorded in response to octyl esters and hexanoates. Octyl hexanoate did not elicit a significant EAG in male antennae.

A3. Female versus Male Antennal Responses. Regarding the five groups of esters tested, none of the acetates showed significant differences in EAGs between female and male antennae. Of the propionates, (*E*)-2-hexenyl propionate and heptyl propionate elicited larger responses in males. Most butanoates also elicited larger relative responses in males, except hexyl butanoate, (*E*)-2-hexenyl butanoate, and (*Z*)-3-hexenyl butanoate, for which there was no difference between sexes. Male antennae responded more to most of the pentanoates as well, although both sexes responded similarly to (*Z*)-3-hexenyl pentanoate, octyl pentanoate, and (*E*)-2-octenyl pentanoate. The hexanoates did not elicit larger EAGs in male antennae. (*Z*)-3-Hexenyl hexanoate even elicited a larger relative EAG response in female antennae.

Plant Volatiles (Figure 2, Table 3)

B1. Responses in Female Antennae. The largest EAGs in female antennae were elicited by (*E*)-2-hexen-1-ol (150 ± 18% mean ± CI) and 1-octen-3-ol (126

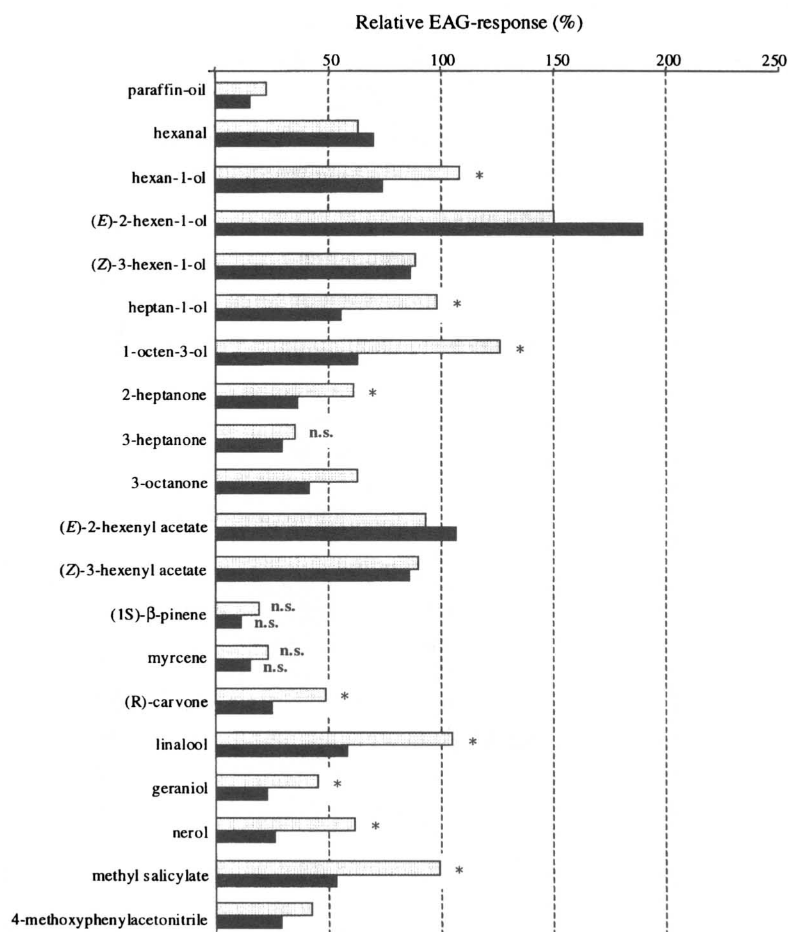

FIG. 2. EAG responses of male and female *L. pabulinus* to the series of plant volatiles. The response bars are based on the backtransformed least-square means. Grey: EAG response of female antennae; black: EAG response of male antennae; n.s.: EAG response not significantly different from the response to the control paraffin oil. *EAG response significantly different between the sexes (after correcting the significance level for multiple comparisons through the Bonferroni method). See text for further explanation.

± 16% mean ± CI). Hexan-1-ol, (*Z*)-3-hexen-1-ol, heptan-1-ol, (*E*)-2-hexenyl acetate, (*Z*)-3-hexenyl acetate, linalool, and methyl salicylate elicited about the same response as the standard (*E*)-2-hexenal. 3-Heptanone, (1*S*)-*β*-pinene, and myrcene did not elicit a significant EAG response in females.

B2. Responses in Male Antennae. The largest EAGs were elicited by (*E*)-

TABLE 3. ANOVA TABLE OF SERIES OF PLANT COMPOUNDS (RESPONSE PROFILES ARE SHOWN IN FIGURE 2)

Tests of fixed effects, source	NDF[a]	DDF[b]	Type III *F*	Pr > *F*	Covariance parameter	Estimate
Gender	1	126	19.96	0.0001	Antenna (gender)	0.3761
Chemical	20	438	81.76	0.0001	Residual	1.4271
Gender × chemical	20	438	6.67	0.0001		

[a]Numerator degrees of freedom.
[b]Denominator degrees of freedom.

2-hexen-1-ol (190 ± 21% mean ± CI). The other alcohols, as well as hexanal, the acetates, methyl salicylate, and linalool elicited ca. 50–100% response relative to the standard (*E*)-2-hexenal. Nerol, (*R*)-carvone, and 4-methoxyphenol acetonitrile elicited smaller EAGs, and the responses of male antennae to (1*S*)-*β*-pinene and myrcene were not different from the control.

B3. Female versus Male Antennal Response. Female antennae showed larger relative EAGs to hexan-1-ol, heptan-1-ol, 1-octen-3-ol, 2-heptanone, (*R*)-carvone, linalool, geraniol, nerol, and methyl salicylate than male antennae.

DISCUSSION

Responses to Esters. Some of the esters tested were <95% pure, hence the recorded EAGs could in part be responses to the corresponding alcohols or, in the case of (*E*)-2-octenyl acetate, to 1-octenyl acetate, since this comprised ~30% of the solution. Despite these inpurities, the five groups of esters showed similar relative response patterns in both *L. pabulinus* male and female antennae. Larger EAGs to the different ester groups could be due to differences in volatilities, i.e., acetates > propionates > butanoates > pentanoates > hexanoates. Since such correlations were not found for any of the ester analogs, the measured EAGs cannot be explained solely by differences in volatilities.

Esters consist of an alcohol and an acid part. With regard to the alcohol part, both sexes were highly sensitive when (*E*)-2-hexenol was present in the esters, i.e., the (*E*)-2-hexenyl esters, and to a lesser extent the hexyl esters, (*Z*)-3-hexenyl esters, and (*E*)-2-octenyl esters. The octyl esters elicited the lowest EAGs. With regard to the acid part, both sexes responded least when hexanoic acids were part of the esters, i.e., the hexanoates, followed by acetates, with largest EAGs to butanoates. Both sexes were most sensitive to (*E*)-2-hexenyl butanoate, which is one of the main compounds found in *L. lineolaris* (Gueldner and Parrot, 1978; Dickens et al., 1995) and is found in both sexes of *L. pabulinus* (F. P. Drijfhout, unpublished result). These results suggest that butanoates may play a

role in the biology of *L. pabulinus*. Whether this role is intra- or interspecific, or attractive or repellent, cannot be concluded from the present data. EAGs only indicate that the insect perceives the volatiles and do not reveal their function in mediating behavior.

Of the esters that elicited larger relative EAGs in male than in female antennae, three are known to play a role in the sexual communication of mirids. Butyl butanoate and (*E*)-2-butenyl butanoate are sex pheromone components in *Campylomma verbasci* (Smith et al., 1991), and (*E*)-2-octenyl butanoate is a sex pheromone component of *Phytocoris relativus* (Millar et al., 1997). In *L. pabulinus*, a possible sexual role of esters was assessed by offering 100 ng of compounds to males in Petri dishes, after which the number of vibrating males was quantified (Groot et al., 1998b). Butyl butanoate elicited male vibration response in 35%, (*E*)-2-butenyl butanoate did not elicit vibration behavior in any, (*E*)-2-hexenyl butanoate elicited a response in 22%, and (*E*)-2-octenyl butanoate elicited a vibration behaviour in 87% (Groot et al., 1998b). Hexyl butanoate was also tested in this assay, since this is the major compound in the metathoracic scent gland of *L. pabulinus*, constituting up to 95% of the total oil (F. P. Drijfhout, unpublished result). When 100 ng of hexyl butanoate was offered, 40% of the males started to vibrate (Groot et al., 1998b). Since this vibration behavior is a specific sexual response of males (Groot et al., 1998a), the compounds that elicited it may play a role in sexual communication in *L. pabulinus*.

Responses to Plant Volatiles. In comparing the relative EAGs, males were less sensitive than females to most of the tested plant volatiles, with the exception of (*E*)-2-hexen-1-ol, to which both sexes showed strong responses. The high response to (*E*)-2-hexen-1-ol may be due to similarity in structure between this fragment and (*E*)-2-hexenyl butyrate, which elicits high EAGs in both sexes as well. On the other hand, (*E*)-2-hexen-1-ol may play a role in general host plant orientation of *L. pabulinus*, as this is a common green leaf volatile (Visser et al., 1979). The nine plant volatiles that showed larger EAGs in female than in male antennae are common plant volatiles (Visser, 1986). 1-Octen-3-ol, linalool, and methyl salicylate are also found in herbivore-infested leaves of several apple cultivars (Takabayashi et al., 1994). Female *L. pabulinus* may use these compounds for host orientation in autumn when they fly back from herbaceous summer hosts to apple orchards to lay winter eggs.

Some common plant volatiles are produced by heteropterans and may play a role in sexual communication (Aldrich, 1988, 1995). In Miridae, these compounds have not been found, but since *L. pabulinus* males are mostly attracted to females on potato leaves (Groot et al., 1996), some plant compounds may be involved indirectly in the attraction between the sexes.

In conclusion, male antennae are relatively more sensitive to a number of insect-produced esters, while female antennae are more sensitive to a number of plant volatiles. The same trend has been seen previously in *L. lineolaris*

males and females (Chinta et al., 1994). This sexual difference in response may be due to the fact that in mirids males are attracted to females, while females may use plant compounds for their orientation towards oviposition sites. Since hexyl butanoate, (*E*)-2-hexenyl butanoate, and (*E*)-2-octenyl butanoate elicited a vibration behavior in some males, these or closely related compounds may be involved in sexual communication, at least at short range. Which of the volatiles that elicited large EAGs play a role in the sexual attraction at long range, remains to be studied.

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CUCURBITACINS: A ROLE IN CUCUMBER BEETLE STEROID NUTRITION?

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Abstract—The conditional role of cucurbitacins as phytosteroid supplements, cholesterol precursors, or ecdysteroid antagonists in the spotted cucumber beetle, *Diabrotica undecimpunctata howardi*, was investigated in two ways: by comparing larval survival and growth rate on cucurbitacin-rich and cucurbitacin-poor squash cultivars of *Cucurbita pepo* and by manipulating the presence of cholesterol, phytosteroids, and cucurbitacins in an artificial diet and examining the effects on adult survival and fecundity. Larvae that developed on cucurbitacin-rich roots grew significantly faster and survived as well as larvae on cucurbitacin-poor roots. There was no evidence, however, that adults could substitute cucurbitacins in vital phytosteroid roles. Beetles reared on a cucurbitacin-rich, phytosteroid-poor diet laid significantly fewer eggs and died significantly younger than beetles with a full complement of dietary phytosteroids and also laid fewer eggs than beetles with no access to phytosteroids in their adult diet. The data are consistent with the hypothesis that, when the side chain of dietary cucurbitacins can be successfully hydrogenated, these compounds play a nutritional role as substitutes or precursors for structural steroids. In contrast, when the carbon–carbon double bond cannot be hydrogenated, cucurbitacins may become antagonists at ecdysteroid receptors, negatively affecting beetle fitness.

Key Words—Cucurbitacins, ecdysteroids, cucumber beetles, *Diabrotica undecimpunctata howardi*, phytosteroids, ecdysteroid antagonist.

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INTRODUCTION

Cucurbitacins are oxygenated tetracyclic triterpenes produced almost exclusively by plants in the family Cucurbitaceae. They are known for their extreme bitterness, cytotoxicity, antifeedant properties, antagonistic action at ecdysteroid receptors, and unexplained ability to stimulate feeding in the adults and larvae of many luperine chrysomelid cucumber beetles (Metcalf et al., 1980; DeHeer and Tallamy, 1991; Miro, 1995; Dinan et al., 1997a).

Cucumber beetles that consume cucurbitacins convert what is not excreted to dihydrocucurbitacin D glucoside by hydrogenation, desaturation, acetylation, and glycosylation (Ferguson et al., 1985; Andersen et al., 1988; Nishida and Fukami, 1990). This compound is then sequestered in the hemolymph, reproductive organs, and exoskeleton (Ferguson et al., 1985; Tallamy et al., 1999), where it conveys some protection from predators (Ferguson et al., 1985) and pathogens (Tallamy et al., 1998). The behavior of cucumber beetles toward cucurbitacins has been described as pharmacophagous because the benefits derived from eating these compounds are assumed to be ecological and not nutritional (Boppré, 1984; Nishida and Fukami, 1990). In fact, it has been suggested that long-term consumption of cucurbitacins may cause a measurable reduction in larval growth rate (Hirsh and Barbercheck, 1996) and adult longevity (Ferguson et al., 1985). Recently, however, cucurbitacin consumption in the absence of ecological stress from predation or infection was found to increase significantly both larval growth rate and adult longevity in the striped cucumber beetle, *Acalymma vittatum* (Fabr.) (Tallamy and Gorski, 1997), suggesting a possible nutritional role for these compounds in beetles that can successfully metabolize them. Understanding all of the ways cucurbitacins physiologically interact with cucumber beetles is a prerequisite to understanding both the ecological costs and benefits associated with such interactions and the evolution of the interactions in the first place.

Moderate amounts of metabolically transformed cucurbitacins might impart nutritional benefits to cucumber beetles as precursors of, or direct substitutes for, structurally or hormonally important steroids. Despite physiological needs for steroids in lipid membranes and as steroid hormone precursors, insects differ markedly from vertebrates in their inability to synthesize steroids de novo from small molecules such as acetate (Clayton, 1964; Downer, 1978; Svoboda and Thompson, 1985). Carnivorous insects overcome this handicap by obtaining cholesterol directly from their food (Rees, 1989), while phytophagous insects use various phytosteroids, either directly for structural requirements, or by first converting them to cholesterol (Bernays, 1994). Lipid membranes require substantial quantities of plant-derived steroids, particularly during periods of growth and development. Cucurbitacin configurations with the proper polarity and binding site activity may be able to substitute directly for such phytosteroids, particularly when dietary sources of useable phytosteroids are limiting.

Hormonal ecdysteroids, in contrast, are usually required in small amounts, but play essential roles in many arthropod physiological processes, including the induction of oocyte meiosis and embryo development (Briers and Hoybrechts, 1984; Schwartz et al., 1985; Lanot et al., 1989), growth (Fristrom et al., 1970, 1985; Mandaron, 1970), molting (Richards, 1981; Bownes and Redfern, 1985; Truman, 1988; Sehnal, 1989), gonadal (Dumser, 1980; Raabe, 1986) and neuronal differentiation (Truman, 1988), and gametogenesis (Hagedorn, 1985, 1989). These hormones are synthesized from cholesterol (Karlson and Hoffmeister, 1963), which, in turn, is derived by most phytophagous arthropods through dealkylation of phytosterols such as campesterol, sitosterol, and stigmasterol (Clayton, 1964; Clark and Bloch, 1959; Svoboda and Thompson, 1985). The pathway of ecdysteroid biosynthesis is complex and involves extensive modification of the cholesterol nucleus (Ikekawa, 1983; Rees, 1989). Substitution with structurally similar homologs at any point in this pathway may represent an energetic savings to the insect. Although cucurbitacins differ from true steroids in that a methyl group is found on carbon 9 rather than on carbon 10 (Lavie and Glotter, 1971; Miro, 1995), similarities between the molecular configuration of cucurbitacins, cholesterol, and ecdysteroids, particularly in ring A and the highly oxygenated side chain (Figure 1), increase the probability that these triterpenes can act as cholesterol precursors requiring only minor biotransformation.

The structural similarities between cucurbitacins and physiologically important steroids may enable cucurbitacins to play an active role in cucumber beetle nutrition, but these same similarities enable cucurbitacins to antagonize ecdysteroids under certain conditions (Dinan et al., 1997a,b). The degree to which cucurbitacins contribute to, or antagonize, beetle fitness may be a function of numerous factors including diet quality, developmental stage, cucurbitacin dose, and/or the length of cucurbitacin exposure. In an initial attempt to identify the relative importance of these factors, we used the spotted cucumber beetle (southern corn rootworm), *Diabrotica undecimpunctata howardi* Barber (Coleoptera; Chrysomelidae, Luperini), to examine the effect of cucurbitacins on larval and adult survivorship, larval growth rate, and female fecundity when dietary sources of phytosteroids and cholesterol were low or absent.

METHODS AND MATERIALS

The ability of spotted cucumber beetles to obtain nutritional benefits from cucurbitacins was investigated in two experiments. In the first experiment, we quantified the effect of dietary cucurbitacins on larval growth rate and survivorship. Because it is very difficult to get cucumber beetle larvae to accept an artificial diet, we compared growth rate and survivorship of larvae on two *Cucurbita pepo* squash cultivars that are similar nutritionally but differ in the cucur-

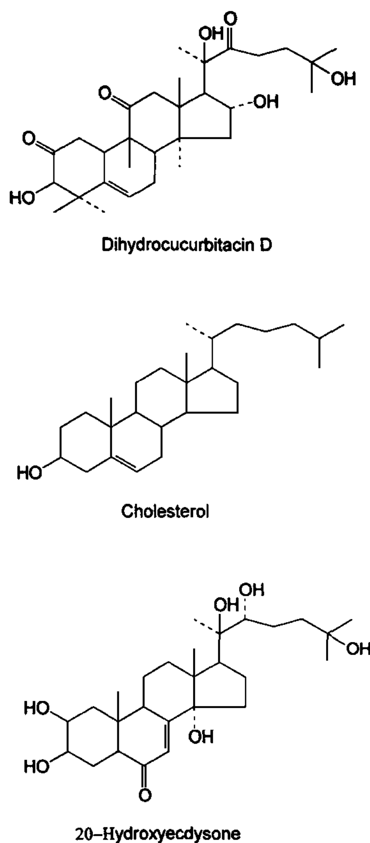

FIG. 1. Structural similarity between dihydrocucurbitacin D, cholesterol, and 20-hydroxyecdysone.

bitacin content of their roots (Tallamy and Gorski, 1997). Spotted cucumber beetle eggs were obtained from the DuPont Co. (Stine-Haskell Laboratory, Newark, Delaware) and reared on root mats sprouted from seeds of *C. pepo* Black Beauty zucchini, a cultivar with roots high in cucurbitacin E (1.153 mg/g dry weight), and *C. pepo* Yellow Crookneck, which has only trace amounts of cucurbitacins in its roots (0.027 mg/g dry weight). Root mats of both cultivars were grown in 32-oz styrofoam cups on several layers of dampened Kimpak paper (Seedburo Equip. Co., Chicago, Illinois). Within 1 hr of hatching, 10 larvae were placed in each of 11 cups bearing roots of each treatment. Cups were then placed in an environmental chamber at 27°C, 15L : 9D photoperiod. The root mats within each cup were moistened as needed. After 12 days, all cups were dismantled

and the larvae were counted and frozen. Frozen larvae from both treatments were dried overnight in a drying oven at 45°C and weighed; the weights were compared by *t* test (SAS Institute, 1989). The percentage of larvae surviving from each cup was arcsine-transformed and compared as a functon of treatment by *t* test.

A second experiment was conducted to examine the effect of cucurbitacins on adult longevity and fecundity in the absence of dietary phytosteroids. Four diet treatments were established, each consisting of 20 male–female pairs. The diets of all treatments were based on the formula of Guss and Krysan (1973). Before diets were prepared, however, all phytosterols and other fats were extracted from the corn meal and wheat germ used in the diets. Approximately 250 g of cornmeal powder (Quaker Oats Co.) and 250 g fiber-rich wheat germ with no sodium (Kretschmer) were Soxhlet extracted for 48 hr with petroleum ether. The defatted powders were air-dried for 3 hr and then oven-dried for 1 hr at 40°C before the diet was mixed and modified for each treatment in the following ways. Diet I consisted of the unmanipulated constituents for spotted cucumber beetle adult diet as recommended by Guss and Krysan (1973). Cornmeal and wheat germ were not extracted prior to use and were supplemented with cholesterol. We included cholesterol in diet I as a supplement to the phytosteroids naturally present in cornmeal and wheat germ because we were attempting to provide an ample supply of all of the steroids that spotted cucumber beetle adults might encounter in their normal phytophagous diet. Diet II was created by amending the defatted standard diet with 12.5 mg of cucurbitacin D per 100 g diet with no cholesterol supplement. Diet II contained an amount of cucurbitacin equal to the sterol called for by Guss and Krysan (1973) in the formulation of a nutritionally balanced artificial diet. A cholesterol-rich diet (diet III) was created by omitting cucurbitacin and adding 12.5 mg of cholesterol (Sigma) to the defatted formulation. Diet IV was comprised of defatted corn meal and wheat germ and contained neither cholesterol, other phytosteroids, nor cucurbitacins. The completed diets were stored in a freezer when not in use. Cucurbitacins used to amend the diet of treatment I were extracted and purified (Halaweish and Tallamy, 1993) from bitter gourds of *C. andreana* grown from seed in a greenhouse.

Beetles used in this experiment were obtained from DuPont Co. as teneral adults that had been reared as larvae on cucurbitacin-free corn roots (Pioneer No. 3397). Each beetle pair was placed in a shallow styrofoam cup (10 cm diam) supplied with: (1) a plastic oviposition cup (4 × 2 cm) fitted with layered damp filter paper, (2) a similar cup containing an ad libitum supply of the appropriate diet (replaced every other day), and (3) a plastic lid with a 2-cm-diam. ventilation hole covered with nylon mesh screening. Beetles obtained water from the damp filter paper in the ovipositional cups. Beetle cups were housed in an environmental chamber at 27°C, 15L : 9D photoperiod. In an attempt to minimize the effects of phytosteroids stored by larvae for use as adults, all beetles were main-

tained on the steroid and cucurbitacin-free diet for the first 20 days of their adult life before fecundity records were initiated. After 20 days, eggs were removed from each container every other day and counted, as were any dead beetles.

Differences among treatments in total fecundity and age at death were detected by analysis of variance (ANOVA) and compared by Ryan's Q-multiple comparison procedure at $P = 0.05$ (SAS PROC GLM; SAS Institute, 1989). Fecundity data were log transformed to correct problems with heteroscedasticity.

RESULTS

Results from larvae and adults were conflicting. Spotted cucumber beetle larvae that were reared exclusively on cucurbitacin-laden roots not only survived as well as larvae reared on roots with traces of cucurbitacins (Figure 2, $t = -0.7122$: $df = 19$, $P = 0.4850$), but also grew significantly larger ($t = 4.9016$: $df = 20.5$, $P = 0.0001$). Larvae reared on *C. pepo* Black Beauty roots had higher growth rates even though these roots contained significantly less nitrogen than nonbitter *C. pepo* Yellow Crookneck roots (Tallamy and Gorski, 1997).

There was no evidence from measures of fecundity or survivorship that adults were able to substitute cucurbitacins for missing phytosteroids. The addition of cucurbitacins to a diet free of phytosteroids (diet II) did not improve female fecundity or adult survivorship in comparison to beetles that had been fed diets I, III, or IV (Figure 3). However, females that ate a diet lacking steroids but rich in cucurbitacin D (diet II) laid significantly fewer eggs than beetles pro-

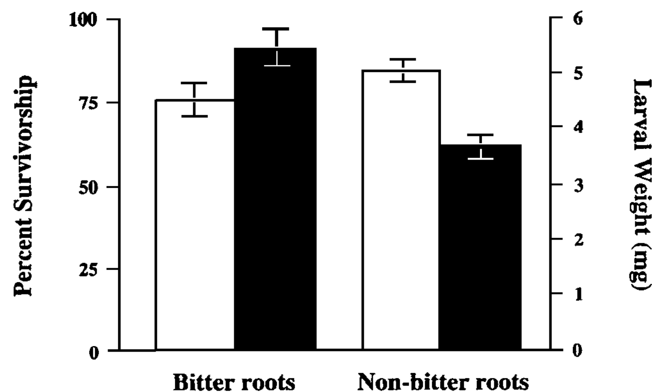

FIG. 2. Effect of dietary cucurbitacins (bitter versus nonbitter roots) on the growth rate (dark bars) and survivorship (light bars) of *Diabrotica undecimpunctata howardi* larvae. Statistical intervals: ±1 SE.

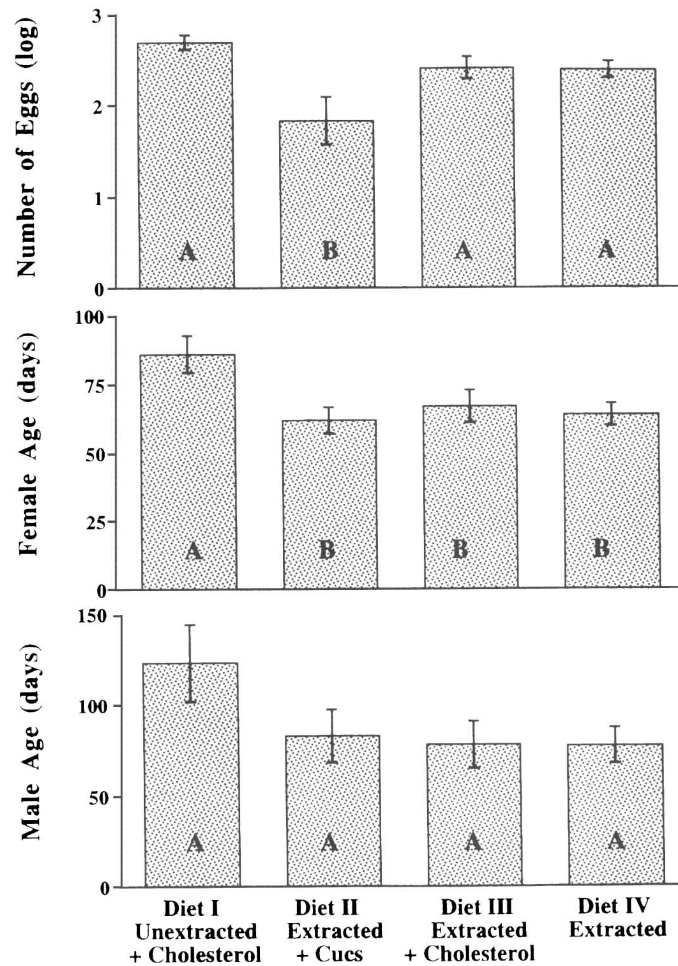

FIG. 3. Effect of cucurbitacins, cholesterol, and dietary phytosteroids on the fecundity and survivorship of *Diabrotica undecimpunctata howardi* adults. Statistical intervals: ±1 SE. Means not sharing the same letter are significantly different (Ryan Q-multiple comparison procedure at $P = 0.05$).

vided with the other three diets (ANOVA: $F = 5.45$; $df = 3, 58$; $P = 0.0024$). Extracted diets (diets II, III, and IV) significantly shortened the life of females relative to the unextracted diet (ANOVA: $F = 4.27$; $df = 3, 58$; $P = 0.0088$), but had only marginal effects on male longevity (ANOVA: $F = 2.22$; $df = 3, 58$; $P = 0.0957$).

DISCUSSION

Manipulations of both larval and adult diets of the spotted cucumber beetle provided circumstantial support for the hypothesis that cucurbitacins can play both positive and negative roles in cucumber beetle nutrition, depending upon the developmental stage and/or the context in which they are consumed. Larvae reared on cucurbitacin-rich squash roots grew significantly faster than those on roots that were nearly cucurbitacin-free. When cucurbitacins are eaten in relatively high doses (≈1 mg/g dry weight) in the larval stage, their metabolites may substitute for or augment naturally occurring phytosteroids in the construction of lipid membranes. It is also possible, however, that larvae grew faster on bitter roots simply because the cucurbitacin phagostimulants caused them to eat faster. This hypothesis does not assume a nutritional role for cucurbitacins, but it does suggest that these compounds can be eaten with impunity while beetles are in the larval stage. Further study is required to discriminate between the nutritional and phagostimulant hypothesis, but evidence from studies of the effects of prior exposure to cucurbitacins suggests that these compounds are phagostimulants for cucumber beetles only on a short-term basis (Tallamy and Halaweish, 1993; Lomberk, 1999). When both larvae and adults are exposed to cucurbitacins for more than a day, their phagostimulatory response all but disappears. Therefore, we consider the long-term phagostimulation that would have been required from cucurbitacin laden roots to account for the 28% increase in larval growth rate to be unlikely.

Benefits postulated to be imparted to larvae from a diet that included cucurbitacins were not realized by adults in our study. Females fed a steroid-poor, cucurbitacin-rich diet laid significantly fewer eggs than females receiving a diet with no phytosteroids, a diet rich in cholesterol only, or a diet rich in several phytosteroids. Females fed the unextracted diet lived significantly longer than beetles with no access to phytosteroids, despite the presence of cholesterol in diet III. Possibly, nonsteroidal nutrients were altered or removed from the wheat-germ and cornmeal components of the diet during extraction with petroleum ether.

The contrary effects of dietary cucurbitacins on the fitness of spotted cucumber beetle larvae (positive or neutral) and adults (negative) may be a function of age-related differences in the ability of cucumber beetles to hydrogenate the active cucurbitacin side chain. Dinan et al. (1997a,b) have shown that, because of their structural similarity to steroidal molecules, cucurbitacins such as B, D, E, I, and F that have a Δ^{23}-22-oxo functional group in the side chain are powerful ecdysteroid antagonists. Insects not capable of hydrogenating this side chain to remove the carbon–carbon double bond (i.e., insects not physiologically adapted to eating cucurbits or adapted cucumber beetles that consume more cucurbitacins than they can hydrogenate) may lose critical activity at ecdysteroid receptors from the antagonistic action of cucurbitacins that retain side-chain activity. This might be particularly damaging in adults during peri-

ods of high ecdysteroid activity such as oogenesis (Hagedorn, 1985) and suggests one reason why adults of several species of cucumber beetles regulate their intake of these compounds (Tallamy and Halaweish, 1993; Tallamy, unpublished data). If, however, the cucurbitacin side chain is able to be effectively hydrogenated to a dihydrocucurbitacin configuration (Andersen et al., 1988; Nishida et al., 1992), the molecule can no longer antagonize ecdysteroid hormones (Dinan et al., 1997a) and may gain function as a steroid substitute. Phytosteroids such as campesterol, sitosterol, and stigmasterol have methyl or ethyl groups in their side chains that must be dealkylated before conversion to cholesterol (Clark and Bloch, 1959; Ikekawa, 1985; Svoboda and Thompson, 1985). The cucurbitacin side chain, however, has no methyl or ethyl groups and thus does not require dealkylation to resemble the side chain of cholesterol. This increases the probability that dihydrocucurbitacins can substitute directly for cholesterol in lipid biostructures or serve as a cholesterol precursor (Figure 1). Since cholesterol usually occurs in plants only in trace amounts, it is common for phytophagous insects to either convert other phytosteroids to cholesterol or to use these phytosteroids directly for the bulk of their structural requirements without first converting them to cholesterol (Svoboda and Thompson, 1985). This may be the mechanism by which large quantities of cucurbitacins are permanently sequestered in cucumber beetle tissues and by which *D. undecimpunctata howardi* and *A. vittatum* larvae grow faster when fed bitter roots (Tallamy and Gorski, 1997).

We conclude by suggesting that when dietary cucurbitacins can be successfully hydrogenated, they may provide nutritional benefits to cucumber beetles in the form of substitutes or precursors for structural steroids. These benefits, however, may be restricted to larval stages because the demand for structural steroids is high in larvae or because larvae are better than adults at completely hydrogenating the active cucurbitacin side chain. Cucurbitacins appear to be detrimental to adult reproductively active females whose need for structural steroids is low but whose use of ecdysteroids during oogenesis is high. These results further emphasize the complexity of the interactions between cucurbitacins, the plants that produce them, and the cucumber beetles that are stimulated to eat them.

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SEQUESTRATION, MAINTENANCE, AND TISSUE DISTRIBUTION OF PYRROLIZIDINE ALKALOID *N*-OXIDES IN LARVAE OF TWO *Oreina* SPECIES

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Abstract—*Oreina cacaliae* and *O. speciosissima* are leaf beetles that, as larvae and adults, sequester pyrrolizidine alkaloid *N*-oxides (PAs) as defensive compounds from their host plants *Adenostyles alliariae* and *Senecio nemorensis*. As in most *Oreina* species, *O. speciosissima* is also defended by autogenously produced cardenolides (mixed defensive strategy), whereas *O. cacaliae* does not synthesize cardenolides and is exclusively dependent on host-plant-acquired PAs (host-derived defense). Adults of the two *Oreina* species were found to have the same PA storage capacity. The larvae, however, differ; larvae of *O. speciosissima* possess a significantly lower capability to store PAs than *O. cacaliae*. The ability of *Oreina* larvae to sequester PAs was studied by using tracer techniques with ^{14}C-labeled senecionine *N*-oxide. Larvae of the two species efficiently take up [^{14}C]senecionine *N*-oxide from their food plants and store the alkaloid as *N*-oxide. In *O. cacaliae*, there is a slow but continuous loss of labeled senecionine *N*-oxide. This effect may reflect the equilibrium between continuous PA uptake and excretion, resulting in a time-dependent tracer dilution. No noticeable loss of labeled alkaloid is associated with molting. Senecionine *N*-oxide is detectable in all tissues. The hemolymph is, with ca. 50–60% of total PAs, the major storage compartment, followed by the integument, with ca 30%. The alkaloid concentration in the hemolymph is approximately sixfold higher than in the solid tissues. The

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selectivity of PA sequestration in larvae is comparable to PA sequestration in the bodies of adult beetles.

Key Words—*Oreina* spp., Coleoptera, Chrysomelidae, alkaloid sequestration, pyrrolizidine alkaloid *N*-oxide, senecionine *N*-oxide, chemical defense, larval defense.

INTRODUCTION

Adult leaf beetles of the Alpine genus *Oreina* are chemically protected by defensive secretions released from exocrine glands located in the elytra and pronotum (Pasteels et al., 1988a, 1994). Chemical defense in *Oreina* is primarily autogenous by de novo synthesized cardenolides (Pasteels et al., 1996; Dobler et al., 1996). Only a few *Oreina* species feeding on plants belonging to the Asteraceae, tribe Senecioneae, sequester pyrrolizidine alkaloid *N*-oxides (PAs) from their host plants (Pateels et al., 1996). Since the first report (Pasteels et al., 1988b), the mechanism of PA sequestration in adults of *Oreina* has been studied in detail (Pasteels et al., 1996; Hartmann et al., 1997). *O. cacaliae* is the only species that does not endogenously produce cardenolides, and instead sequesters PAs from its host plants, *Adenostyles alliariae* and *Senecio nemorensis*. In beetles, plant-acquired PAs are taken up into the hemolymph. Absorbed PAs are stored in the body, from where they are partly translocated into the secretory glands and released with the defensive secretions. Thus, in beetles, PAs are stored in two different compartments, the body (i.e. hemolymph and integument) and the glands (Rowell-Rahier et al., 1991; Ehmke et al., 1991; Pasteels et al., 1992, 1995; Hartmann et al., 1997).

Unlike adults, *Oreina* larvae do not have any mechanical protection and do not possess defensive glands. However, they are chemically defended. They autogenously produce cardenolides, which they store in their bodies (Eggenberger and Rowell-Rahier, 1993; Dobler and Rowell-Rahier, 1994). This is remarkable, since in adults of *Oreina* species, cardenolides have not been detected in any tissue except the glands and, in oviparous species, the eggs (Dobler and Rowell-Rahier, 1994; Pasteels et al., 1996). Species that sequester PAs from their host plants as adults, such as *Oreina cacaliae*, *O. elongata*, *O. intricata*, and *O. speciosissima* (Pasteels et al., 1995, 1996; Hartmann et al., 1997), also sequester PAs as larvae (Dobler and Rowell-Rahier, 1994).

In this study, we analyzed the capability and capacity of larvae and adults of *O. cacaliae* and *O. speciosissima* to sequester PAs from their two PA-containing food plants, *A. alliariae* and *S. nemorensis*. Subsequently, tracer feeding experiments with radioactively labeled senecionine *N*-oxide were performed to study alkaloid uptake, maintenance, and tissue distribution in larvae. The tracer techniques had been successfully applied in the past to study PA uptake and metabolism in *Oreina* adults (Ehmke et al., 1991; Pasteels et al., 1992).

METHODS AND MATERIALS

Insects. For chemical analysis, adults of *O. cacaliae* (Schrank) and *O. speciosissima* (Scopoli) were collected at Tschiertschen (Graubünden, Switzerland) at elevations of 1800–2000 m (*O. cacaliae*) and 1500–1800 m (*O. speciosissima*) feeding on *Adenostyles alliariae* (Gouan) Kern. and *Senecio nemorensis* L. (s. str.) (Asteraceae, Senecioneae). In the laboratory, the beetles were kept on their food plants at room temperature or in a cool-chamber at 8°C until use. The offspring were raised on *A. alliariae* and *S. nemorensis.*

For tracer experiments, *O. cacaliae* adult females were collected in Appenzell (Switzerland) at elevation of 1300 m on *A. allariae* and *O. speciosissima* adult females in Zastler (Black Forest, Germany, 1170 m) on *Petasites paradoxus*, the preferred larval food plants in the field. *P. paradoxus* does not contain PAs in its leaves. Larvae produced by these females were kept on their respective food plants at 17°C until they reached the larval stage required in the experiment.

Tracer Feeding Experiments. [^{14}C]Senecionine *N*-oxide (1.07 GBq/mmol) was prepared biosynthetically from [1,4-^{14}C]putrescine (4.4 GBq/mmol, Amersham Buchler, Braunschweig, Germany) by using root cultures of *Senecio vulgaris* and was subsequently purified (Hartmann, 1994). [^{14}C]Senecionine *N*-oxide prepared by this method was chemically and radiochemically pure. For the feeding experiments, 10 μl of a methanolic solution of the tracer (ca. 2 kBq corresponding to ca. 3000 cps) was painted on the surface of leaf disks (6–10 mm diameter depending on the larval age) prepared from fresh leaves of *A. alliariae* (*O. cacaliae*) and *P. paradoxus* (*O. speciosissima*). After evaporation of the solvent, the disks were placed in Petri dishes (5 cm diameter) with one larva (last instar) each. Larvae were allowed to feed 24 hr. After 24 hr, larvae that had eaten less than half of the leaf offered were not considered. The remaining individuals were transferred to fresh untreated leaves and allowed to feed until termination of the experiment.

Individual larvae were extracted twice with 2 ml methanol. After centrifugation, total radioactivity was determined by scintillation counting (Rialuma, Baker). The remains of the tracer leaves were collected and analyzed. Total radioactivity evaluated for larval extracts was related to the amount of "ingested tracer." Ingested tracer (100%) is defined as total radioactivity offered minus total radioactivity recovered from the remains of the tracer-treated food leaf.

Separation of the labeled extracts to localize senecionine *N*-oxide and any of its metabolites was achieved by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) according to Ehmke et al. (1991) and Hartmann and Dierich (1998). Radioactively labeled compounds were located by means of a TLC multichannel analyzer (Rita-32a, Raytest) and a HPLC radioactivity monitor LB-506D (Berthold).

Dissection. The integument of third- and fourth-instar larvae was dorsally opened with small scissors and the hemolymph was collected in glass capillary tubes and stored in methanol until analysis. Subsequently, the gut and the fat body were prepared and immediately preserved in methanol. The residual tissue was then called integument.

Gas Liquid Chromatography (GLC). The true PA pattern of the food plant leaves as well as that of the *Oreina* larvae and adults was qualitatively and quantitatively evaluated as described by Witte et al. (1993) and Pasteels et al. (1995). The identity of individual alkaloids was confirmed by combined gas chromatography–mass spectrometry (GC-MS) (Witte et al., 1993; Hartmann et al., 1997).

RESULTS

PA Contents and Patterns in Larvae and Adults. Field-collected adults of *O. cacaliae* and *O. speciosissima* and their offspring were raised on their two food plants *Senecio nemorensis* and *Adenostyles alliariae* in the laboratory and were then analyzed for their total PA contents and concentrations (Table 1). The amount and concentration of PAs sequestered by either larvae (analyzed after the last larval molt) or adults of the two species is not affected by the food-plant species, although the two species contain structurally different PAs (Figure 1) (Hartmann et al., 1997). On the two food plants, larvae but not adults of *O.*

TABLE 1. AMOUNTS AND CONCENTRATIONS OF TOTAL PAs SEQUESTERED BY LARVAE AND ADULTS OF *Oreina cacaliae* AND *O. speciosissima* ON TWO HOST PLANTS[a]

			Total PAs (μg ± SD)	
	N	Host plant	per individual	per gram/g fresh weight
Oreina cacaliae				
Larvae	19	*S. nemorensis*	77.7 ± 31.7[1]	2031 ± 891[2]
Adults	9	*S. nemorensis*	74.6 ± 12.6	909 ± 101
Larvae	19	*A. alliariae*	68.8 ± 25.8[3]	1700 ± 587[4]
Adults	9	*A. alliariea*	98.0 ± 6.1	1187 ± 96
Oreina speciosissima				
Larvae	16	*S. nemorensis*	12.2 ± 6.3[1]	428 ± 169[2]
Adults	4	*S. nemorensis*	51.5 ± 16.3	797 ± 76
Larvae	13	*A. alliariae*	13.0 ± 6.6[3]	339 ± 167[4]
Adults	2	*A. alliariae*	94.0	1465

[a]The PA contents and concentrations of *O. cacaliae* and *O. speciosissima* feeding on the respective host plants are significantly different; Student's *t* test, [1,2]$P < 0.005$; [3,4]$P < 0.01$.

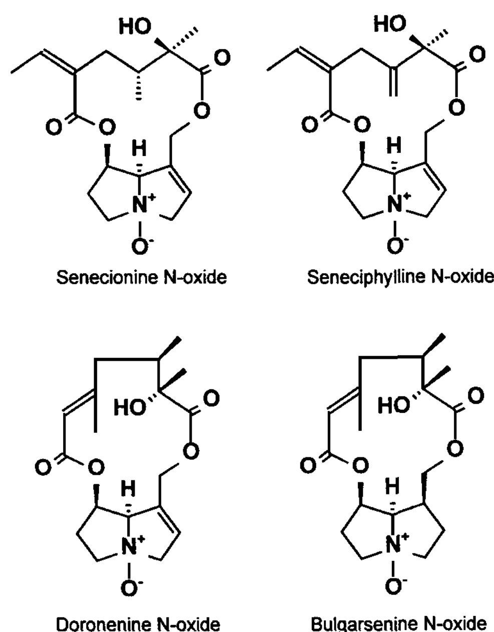

FIG. 1. Major PAs sequestered by *Oreina* larvae from their host plants *Adenostyles alliariae* (*N*-oxides of seneciphylline and senecionine) and *Senecio nemorensis* (*N*-oxides of doronenine and bulgarsenine).

cacaliae showed a higher capacity (Student's *t* test, $P < 0.005$, *S. nemorensis*; and $P < 0.01$, *A. alliariae*) to sequester PAs than larvae of *O. speciosissima* (Table 1).

Seneciphylline *N*-oxide is the major PA found in larvae and adults feeding on *A. alliariae*, and it is occasionally accompanied by trace amounts of senecionine *N*-oxide. *Oreina* larvae and adults feeding on *S. nemorensis* contain doronenine *N*-oxide (65–85%) and bulgarsenine *N*-oxide (15–35%) as major alkaloids (data not shown).

Sequestration of [^{14}C]Senecionine N-*Oxide.* Larvae (fourth instar) of the two *Oreina* species were pulse-fed with [^{14}C]senecionine *N*-oxide for 24 hr and then transferred to untreated host plant leaves. The percentage of ingested radioactivity absorbed was assayed 24–96 hr (*O. cacaliea*) and 24–72 hr (*O. speciosissima*) following termination of the tracer pulse-feeding. Larvae of the two species efficiently sequester the labeled senecionine *N*-oxide. Approximately 20–30% of the ingested alkaloid is absorbed into the body (Figure 2). In *O. cacaliae*, the sequestered labeled alkaloid *N*-oxide is lost over time, whereas it remains stable in *O. speciosissima*. A two-factor ANOVA with radioactivity

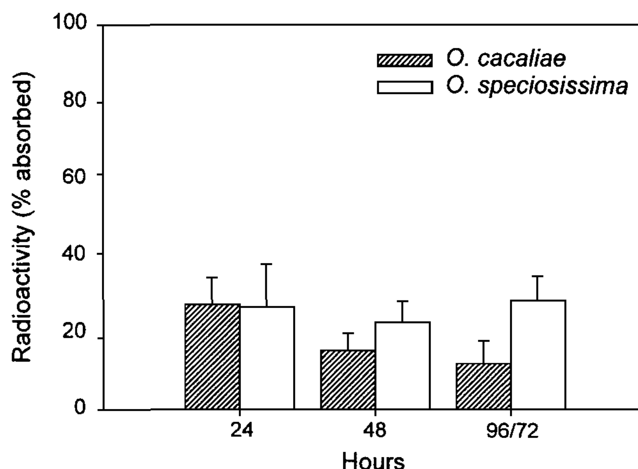

FIG. 2. Sequestration of [^{14}C]senecionine *N*-oxide by larvae (fourth instar) of *O. cacaliae* and *O. speciosissima*. Total ingested radioactivity was set 100%. Number of individuals analyzed at the time indicated, *N* 11–12, mean % ± SD. The tracer (3000 cps) was applied on leaf disks and offered to single larvae. After 24 hr, larvae were removed and allowed to continue feeding on untreated leaves for another 24, 48, and 96 (*O. cacaliae*) or 72 hr (*O. speciosissima*) as indicated.

recovered as dependent variable, revealed an effect of the factor time ($F_{2,61}$ = 8.259; $P = 0.001$), of the factor species ($F_{1,61}$ = 21.384, $P < 0.001$) as well as an interaction ($F_{2,61}$ = 8.409, $P = 0.001$). The mean radioactivity recovered from *O. speciosissima* larvae (26.16, SD = 7.97, $N = 33$) is higher than that of *O. cacaliae* larvae (18.73 ± 8.79, $N = 34$).

TLC and HPLC analysis of larval methanol extracts revealed that most of the soluble radioactivity was recovered as senecionine *N*-oxide (>85%). Only a small proportion of the sequestered radioactivity was recovered as a polar metabolite (Hartmann et al., 1999). Labeled senecionine was either completely absent or detectable in traces only.

In a long-term experiment, young (i.e. one day after molting) second and third instars of *O. cacaliae* were fed [^{14}C]senecionine *N*-oxide. After 24 hr, larvae were transferred to untreated host-plant leaves and analyzed after time intervals as indicated in Figure 3. The two instars transfer the sequestered alkaloid to the next instar. However, larvae apparently lose sequestered [^{14}C]senecionine *N*-oxide during their development. At the end of the fourth instar, larvae had lost by excretion more than 80% of the alkaloid *N*-oxide sequestered at the beginning of the third instar (Figure 3). No obvious loss of alkaloids could be observed during molting. In a two-factor ANOVA with recovered radioactivity as de-

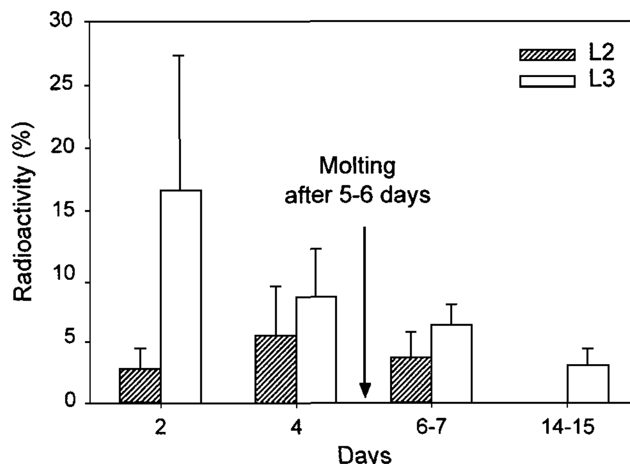

FIG. 3. Retention and loss of ingested [^{14}C]senecionine *N*-oxide during growth of *O. cacaliae* larvae. Larvae at the beginning of the second (L2) and third (L3) instars were pulse fed with labeled alkaloid (3000 cps each). After 24 hr, larvae were removed and allowed to continue feeding on untreated leaves. Ingested radioactivity was set 100%. Number of samples analyzed at each time: N = 4–6; mean % ± SD.

pendent variable, there is a significant effect of the factor larval instar ($F_{1,25}$ = 15.83, P = 0.001), but not of the factor time ($F_{2,25}$ = 2.136, P = 0.14) up to days 6–7. The interaction was not significant ($F_{2,25}$ = 2.92, P = 0.07). On average, third instars (10.48 ± 6.68, N = 18) retain more radioactivity than second instars (4.19 ± 2.78, N = 18). In third instars, the decrease of radioactivity with time becomes significant if amounts at days 14–15 are included in the analysis ($F_{3,15}$ = 4.3900, P = 0.0209).

Distribution of Sequestered [^{14}C]Senecionine N-*Oxide Between Larval Tissues.* Radioactively labeled alkaloid *N*-oxide was detected in all major larval tissues (Figure 4). Almost 50–60% of total senecionine *N*-oxide was found in the hemolymph, followed by the integument (27–31%). Fat body and gut contained less than 8% each. Only traces of radioactivity (<3%) were associated with the exuvia; this corresponds well with the observation that no loss of radioactivity was observed during molting (Figure 3). A rough calculation of the tissue concentrations revealed almost the same concentrations in the solid tissues and an approximately sixfold higher concentration in the hemolymph, the major PA storage compartment. The solid tissues are necessarily contaminated with hemolymph; therefore, the total amount and concentrations measured for these tissues are overestimated. This stresses the importance of the hemolymph as a major storage compartment.

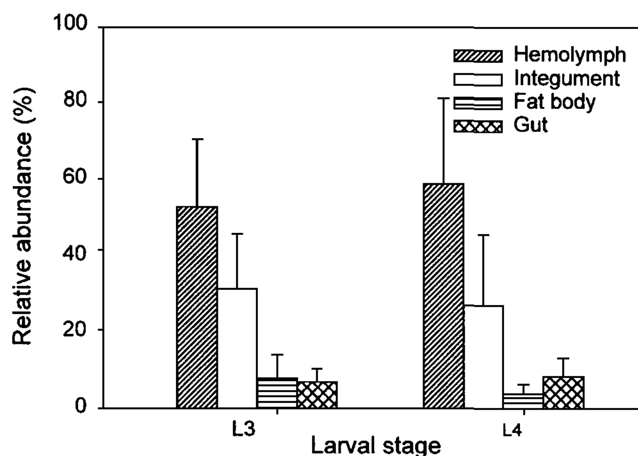

FIG. 4. Distribution of radioactivity associated with hemolymph and different solid tissues of *O. cacaliae* larvae. L3 and L4 larvae were individually fed with [^{14}C]senecionine *N*-oxide (3000 cps each). After 24 hr, larvae were removed and allowed to continue feeding for another 24 hr and were then dissected. Mean % ± SD (*N* = 4 in each series).

DISCUSSION

The amount and concentrations of PAs sequestered in the bodies of larvae and adults of *O. cacaliae* and *O. speciosissima* are of the same order of magnitude as reported recently by Rowell-Rahier et al. (1991), Dobler and Rowell-Rahier (1994), and Pasteels et al. (1996). In comparison to *O. cacaliae*, the PA storage capacity of *O. speciosissima* is less efficient, especially in the larval stage. This may reflect different defensive strategies of the two species: host-derived defense in *O. cacaliae* and mixed defensive strategy in *O. speciosissima* (Dobler and Rowell-Rahier, 1994; Pasteels et al., 1995, 1996).

No significant qualitative differences in the alkaloid patterns were observed between larval and adult populations feeding on the two host plants *A. alliariae* and *S. nemorensis*. Larvae and adults feeding on *A. alliariae* preferentially sequester seneciphylline *N*-oxide, the major plant PA. Larvae and adults feeding on *S. nemorensis* sequester doronenine *N*-oxide, accompanied by a smaller proportion of its 1–2 saturated derivative bulgarsenine *N*-oxide (Figure 1). The preferential sequestration of the 1–2 unsaturated doronenine *N*-oxide is remarkable because this alkaloid is a minor component in *S. nemorensis*, which contains bulgarsenine *N*-oxide as the major alkaloid (Pasteels et al., 1996; Hartmann et al., 1997).

PA sequestration in larvae is efficient and specific. Radioactively labeled

senecionine *N*-oxide is sequestered as *N*-oxide and is almost completely retained in the body. Only a small proportion of the *N*-oxide is transformed into a polar metabolite that recently was identified as a senecionine *O*-glycoside (Hartmann et al., 1999). The sequestered alkaloid *N*-oxide is retained during molting. There is, however, a continuous slow loss during larval growth (Figure 3). This loss was also observed in the short-term experiment illustrated in Figure 2; it involves *O. cacaliae*, not *O. speciosissima*. This seems to contradict the fact that *O. cacaliae* is the species with the more advanced sequestration strategy and a higher PA storage capacity. Here, we have to take into consideration that we are discussing the results of pulse-feeding tracer experiments. *O. cacaliae* larvae feeding on their PA-containing food plant *A. alliariae* have a high systemic load of PAs. This load, defined by the PA storage capacity of the larvae, should be characterized by a well-tuned balance between PA absorption and excretion. The intake of new PAs should be balanced by continuous PA excretion. This, in turn, would result in a continuous dilution of the population of labeled PAs sequestered during the feeding pulse. With *O. speciosissima*, we have a different situation: The larvae fed on their preferred food plant *P. paradoxus*, which does not contain PAs in its leaves, and consequently they are devoid of PAs. During the feeding pulse with labeled senecionine *N*-oxide, larvae only ingest the labeled PAs, which represent a small absolute amount, i.e., <0.5 μg senecionine *N*-oxide per larva. Most likely the PA threshold concentration in the hemolymph will not be reached. As a consequence, the population of sequestered labeled senecionine *N*-oxide remains trapped in the larva's body. Although this interpretation appears to be reasonable, further kinetic studies are needed to confirm it.

The preferential storage of the labeled senecionine *N*-oxide in the hemolymph and a low but almost equal concentration in the solid organs of larvae indicate the absence of specific storage compartments. Here, larvae behave like adult beetles (Pasteels et al., 1992; Hartmann et al., 1997). A similar nonspecific tissue distribution of sequestered compounds, which appears to depend on the chemical polarity of the constituent, has been reported for the tissue distribution of plant-acquired cardenolides in the monarch butterfly (Brower et al., 1988). Adults and larvae of PA-sequestering *Oreina* species are able to sequester PA *N*-oxides from their host plants in a relatively nonspecific manner. Adults transfer PA *N*-oxides from the hemolymph into the exogenous glands where the alkaloid *N*-oxides reach concentrations up to 0.3 mol/liter (Rowell-Rahier et al., 1991; Hartmann et al., 1997), which is 50- to 100-fold higher than the concentrations calculated on the fresh-weight basis for larvae and adults in this study (see Table 1). Phylogenetic studies indicate that *O. cacaliae* switched from autogenous defense by cardenolides to PA sequestration (Dobler et al., 1996; Pasteels et al., 1996; Hsiao and Pasteels, 1999). One may speculate that a switch from autogenous defense to host-plant-acquired PA *N*-oxides occurred in two steps. First, larvae and adults attained the ability to sequester PA *N*-oxides and store

them in their bodies. Second, adult beetles attained the ability to transfer PA *N*-oxides from the hemolymph into the gland cells, where they are concentrated and excreted with the defensive secretion. In comparison to body sequestration, gland sequestration is a more specific and selective process. A number of PA *N*-oxides that are found in the bodies of larvae and adults are never transferred into the glands. For instance, adults of *O. cacaliae* that feed on *S. nemorensis* sequester in their bodies the *N*-oxides of bulgarsenine and doronenine, but transfer only the potentially toxic doronenine into the glands (Pasteels et al., 1996; Hartmann et al., 1997). To obtain more support for the idea of a two-step adaptation to host-derived defense in leaf beetles it would be interesting to see whether there are *Oreina* species sequestering PAs in their bodies but still incapable of transferring them into the secretions.

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IS IAA THE MAJOR ROOT GROWTH FACTOR SECRETED FROM PLANT-GROWTH-MEDIATING BACTERIA?

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Abstract—The phytotoxic or promoting effect of bacterial secretions on root growth of young lettuce seedlings (*Lactuca sativa*) was measured under axenic conditions. It was assumed that the inhibitory or promoting effects of either deleterious rhizobacteria (DRB) or of plant growth promoting rhizobacteria (PGPR) were auxin mediated. To avoid measurements of either parasitism or competition, seedlings were placed adjacent to bacterial colonies, with no contact between the organisms. Auxin excretion rate, evaluated by thin-layer chromatography (TLC) combined with Salkowski's reagent, indicated that all bacteria examined produced and released indole-3-acetic acid (IAA). High levels of IAA (76.6 μM) were excreted by four DRB (*Micrococcus luteus*, *Streptoverticillium* sp., *Pseudomonas putida*, and *Gluconobacter* sp.) during 84 hr of incubation. High concentrations of IAA released by DRB accounted for the suppression of root growth. Other unidentified fractions in the eluates of DRB also inhibited root elongation, but to a lesser extent. Like DRB, four isolates of PGPR (*Agrobacterium* sp., *Alcaligenes piechaudii*, and two different strains of *Comamonas acidovorans*) secreted IAA, but at lower levels (16.4 μM during a similar period of incubation). PGPR secreted growth promoting substances other than IAA, and these are now being investigated.

Key Words—Phytotoxicity, deleterious rhizobacteria, indole-3-acetic acid, lettuce, *Lactuca sativa*, plant-growth-promoting rhizobacteria.

INTRODUCTION

Interactions between microorganisms and higher plants in soil occur mainly in the rhizosphere. Root-colonizing bacteria may influence root growth. Their neg-

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ative or positive effects on plant growth have been studied in ecological and agricultural ecosystems. Allelopathic microorganisms were suggested to suppress annuals adjacent to shrubs, e.g., *Adenostoma fasiculatum* (Kaminsky, 1981), *Coridothymus capitatus* (Katz et al., 1987). Reduction of crop yields associated with "soil sickness", inappropriate crop rotation (Schippers et al., 1987; Bakker et al., 1987), or "replant disease" (Catska et al., 1982; Waschkies et al., 1994) have been, among other factors, related to deleterious soil-borne bacteria. Soil fumigation or solarization is often effective in promoting yields a result of reduction of deleterious and/or pathogenic microorganisms (Gamliel and Katan, 1991). The positive effect of plant-growth-promoting rhizobacteria (PGPR) has been studied on annual crops, such as wheat, soybean, lettuce, beans, maize, and barley (Kloepper et al., 1990) and, on a limited scale, on woody species such as apples (Caesar and Burr, 1987) and Douglas fir (Chanway and Holl, 1993).

Up to 80% of rhizobacteria can synthesize indole-3-acetic acid (IAA) (Loper and Schroth, 1986). Root growth promotion by free living PGPR, e.g., *Alcaligenes faecalis*, *Enterobacter cloacae*, *Acetobacter diazotrophicus*, species of *Azospirillum*, *Pseudomonas*, and *Xanthomonas*, as well as by symbionts, such as *Bradyrhizobium japonicum* and *Rhizobium* spp., has been related to low levels of IAA secretion (Patten and Glick, 1996). In contrast, the inhibitory effect of some deleterious rhizobacteria (DRB) has been related to their high amounts of IAA excretion, e.g., *Enterobacter taylorae* (Sarwar and Kremer, 1995) and *Pseudomonas putida* (Xie et al., 1996).

In this study, we tested the hypothesis that DRB or PGPR inhibit or promote root elongation, respectively, because of different concentrations of IAA excretion. The data are discussed in terms of ecological significance of IAA concentration on root growth of higher plants associated with DRB and PGPR.

METHODS AND MATERIALS

Tests were conducted with eight different bacterial isolates (4 DRB and 4 PGPR). IAA in the bacterial eluates was characterized by thin-layer chromatography (TLC), and the concentration of IAA in the bacterial eluates was measured by using Salkowski's reagent. To evaluate the role of IAA in root growth promotion or inhibition, bacterial eluates were fractionated and the effect of the different fractions on root length of lettuce seedlings was determined.

Isolation of Root Growth Mediating Bacteria. Bacteria were isolated from the rhizoplane and the rhizosphere of stocks of *Rosa indica* grown in tuff (volcanic scoria). Root segments (3 mm diam., 2 cm long) were shaken for 60 min in a 5-ml dilution medium (0.1% agar, 1 ml phosphate buffer, 800 ml distilled water) on a vortex mixer. Soil samples (1 g dry weight), collected 20 cm away from the root system, where shaken in a 50-ml dilution medium for 30 min. By the dilution plating technique, samples of different volumes were applied to Petri

dishes with melted modified malt yeast agar (2.5 g/liter malt extract; 4.0 g/liter yeast extract; 1 ml/liter $CoCl_2$; 15 g/liter bacto-agar). After seven days of incubation (27°C), single colonies from the dilution plates were streaked onto malt yeast agar (MYA; 10.0 g/liter malt extract; 4.0 g/liter yeast extract; 1 ml/liter $CoCl_2$; 15 g/liter bacto-agar). Selected bacterial isolates were identified to the species level by their fatty acid pattern with gas chromatography according to Sasser (1990).

Seeds of domesticated lettuce (*Lactuca sativa* cv. Noga 936) exhibiting high rates of germination and less variability in germination or growth were used as test plants. Seeds were surface sterilized in 2.25% sodium hypochloride for 6 min, followed by repeated washing with 500 ml of sterile distilled water for another 6 min, air dried, and stored at 5°C. To study the phytotoxic or promoting effect, each isolate was plated on 1/5 of the area of each of four Petri dishes (9 cm diam.) containing MYA (Figure 1). After 48 hr of incubation at 27°C, 10 surface-sterilized seeds of lettuce were placed 1 cm away from the colony line. Plates containing the seeds and bacterial isolates were placed vertically for an additional 48 hr incubation (25°C; fluorescent light, 130 μmol photons/m^2/sec), following which the lengths of roots were measured. Root inhibition (RI) was calculated by $RI = (T/C - 1) \times 100$, while the rate of stimulation (RS) was obtained by $RS = (1 - C/T) \times 100$ (T is root length; C is root length in noninoculated, control plates). Because direct contact between the tested plant and the microorganism was avoided, only the effect of bacterial secretions on root growth was evaluated (Figure 1). Moreover, because young lettuce seedlings (24–48 hr after wetting), whose growth is supported by storage tissue, were used as test plants, inhibition by competition between the bacteria and the seedlings was prevented. Bacterial isolates were characterized by their potential to promote or inhibit root growth of lettuce seedlings.

Determination of IAA in Bacterial Secretions. Single bacterial colonies were inoculated in liquid malt yeast medium amended with 0.2 g/liter L-tryptophan (ca. 1 mM) and grown for 84 hr at 27°C on an orbital shaker (100 rpm). Eventually, bacterial cells were separated from the supernatant by centrifugation for 10 min at 13,000 rpm, and supernatants were acidified to pH 2.5–3.0 with 1 N HCl. The supernatant (30 ml) was extracted twice with 60 ml ethyl acetate, and subsequently ethyl acetate was evaporated to dryness in a rotoevaporator. The extracts were dissolved in 300 μl methanol and kept at −20°C. The ethyl acetate extracts (10–20 μl) were plated on TLC plates (20 × 20 F254, 0.2 mm, Merck) and developed in either ethyl acetate–isopropanol–ammonium hydroxide (45 : 35 : 20) or in ethyl acetate–chloroform–formic acid (55 : 35 : 10). Spots with R_f values identical to authentic IAA were identified under UV light (254 nm) or by spraying the plates with Ehmann's reagent (Ehmann, 1977) consisting of solutions A and B at a 3 : 1 ratio: A, 50 ml absolute ethanol, 50 ml concentrated HCl, 1 g *p*-dimethyl aminobenzaldehyde; B, 2.03 g $FeCl_3 \cdot 6H_2O$, 500 ml H_2O, and 300 ml concentrated H_2SO_4.

To determine the effect of bacterial IAA on lettuce root growth, eight different isolates were selected, four of PGPR and four of DRB. Auxin concentration in the culture medium of these isolates was measured colorimetrically with Salkowski's reagent (50 ml 35% perchloric acid; 1 ml 0.5 M $FeCl_3$) (Loper and Schroth, 1986). Ethyl acetate extracts (20 μl) of the culture medium of each of the eight strains were spotted and developed on a TLC plate (20 × 20 F254, 0.2 mm, Merck). Spots with R_f values identical to those of authentic IAA were recovered from the TLC plate and dissolved in 1 ml absolute ethanol, followed by 2 min of vortexing. The silica was separated from the extract by centrifugation for 4 min (13,000 rpm). One ml of Salkowski's reagent was added to the ethanolic extract. Absorbance at 530 nm in a spectrophotometer (Ultrospec 2000, Pharmacia Biotech) was measured after 30 min. After applying Salkowski's reagent, IAA was marked by the appearance of a pink-red color. A standard absorbtion curve was obtained from authentic IAA (Sigma) dissolved in absolute ethanol at different concentrations.

Isolation of Allelopathic Fractions in Bacterial Secretions. Ethyl acetate extracts of the media of selected bacterial isolates were developed on TLC plates (in ethyl acetate–chloroform–formic acid). Spots were detected and marked under UV light (254 nm). Each plate was divided into five segments (R_f 0–0.2, 0.2–0.4, 0.4–0.6, 0.6–0.8, 0.8–1.0), and the silica was separated and eluted in 1 ml absolute ethyl alcohol. Samples were shaken on a vortex (for 3 min) and eventually centrifuged for 5 min (13,000 rpm). To examine the effect of each fraction on root growth, 200 μl of the TLC segment extracts was added to each of five Petri dishes (5 cm diam.) containing one filter paper (Whatman No. 1). After evaporation of ethanol, 0.7 ml of sterilized distilled water was added to the dishes. In each dish, five surface-sterilized lettuce seeds were germinated. Root length was measured 48 hr after germination. The effect of each fraction on root growth was compared to a control (A: elution from an additional 'clean' spot from the TLC plate, in absence of bacterial secretion, hereby termed TLC control; and B: distilled water only, after the evaporation of an equal amount of ethanol, but in the absence of bacterial secretions and solvents).

Root growth in response to different concentrations of authentic IAA was also tested. Authentic IAA, dissolved in ethanol (95%), was diluted in distilled water, and 2 ml of different concentrations (1×10^{-5}–1.0 μM), was applied to four Petri dishes (9 cm diam.) containing Whatman No. 1 filter paper. Each dish contained eight lettuce seedlings, 24 hr after germination. Petri dishes were set vertically for an additional 48 hr, and the effect of various concentrations of authentic IAA on root elongation was measured compared to control seedlings in distilled water with ethanol.

Data Analysis. Data were analyzed statistically by using one- or two-way ANOVA calculated by statistical Excell software (Microsoft) or SPSS software (SPSS Inc.). Correlations were tested by Excell software.

TABLE 1. IAA CONCENTRATION[a] IN BACTERIAL SECRETIONS IN SPOTS RECOVERED FROM TLC PLATES AT $R_f = 0.35$

	PGPR	IAA (μM)		DRB	IAA (μM)
a	*Agrobacterium* sp.[b]	51.2 ± 1.0	*e*	*Micrococcus luteus*	195.1 ± 0.2
b	*Alcaligenes piechaudii*	3.7 ± 0.1	*f*	*Streptoverticillium* sp.[b]	116.9 ± 0.6
c	*Comamonas acidovorans* 26	3.6 ± 0.1	*g*	*Gluconobacter* sp.[b]	22.3 ± 0.1
d	*Comamonas acidovorans* 30	7.1 ± 0.1	*h*	*Pseudomonas putida*	11.7 ± 0.9

[a]IAA concentrations were calculated from the absorbtion of four different samples (see Methods).
[b]Levels of identification were under 40% certainty.

RESULTS

Root-Growth-Mediating Bacteria. Of the eight selected bacterial isolates, five were identified to species (according to Sasser, 1990), at a level exceeding 40% of certainty; identification of the other isolates was uncertain (Table 1).

The effect of DRB is exemplified by *Streptoverticillium* sp. (Figure 1). After 48 hr of incubation, isolates of DRB reduced lettuce root length by 34.6–62.2%, whereas the PGPR isolates promoted root growth by an average of 49.6% (Table 2).

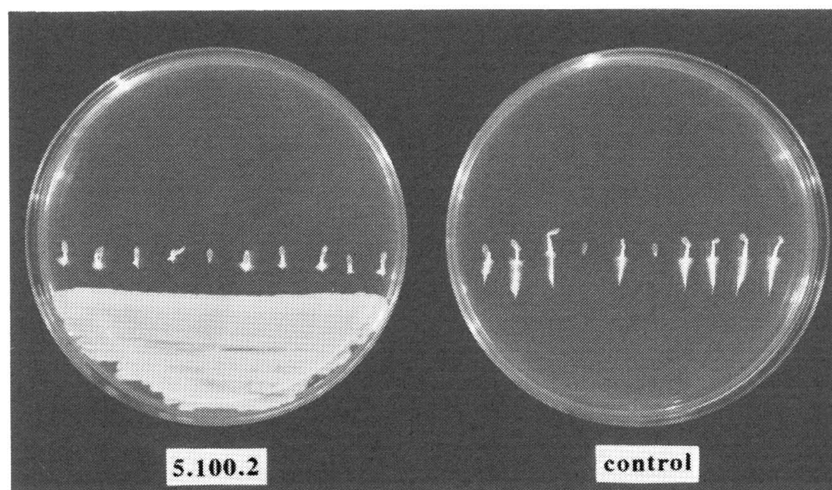

FIG. 1. The effect of a deleterious rhizobacterium, *Streptoverticillium* sp. (5.100.2), on elongation of roots of lettuce seedlings (*Lactuca sativa*), 48 hr after seeds were set to germinate.

TABLE 2. EFFECT OF FRACTIONATED ELUATES OF DRB AND PGPR ON ROOT ELONGATION OF LETTUCE SEEDLINGS (*Lactuca sativa*), COMPARED TO EFFECT OF WHOLE ELUATE (RI, RS)

		DRB[b]							
		Pseudomonas putida		*Gluconobacter* sp.		*Streptoverticillium* sp.		*Micrococcus luteus*	
Fraction No.	*Rf*[a]	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*
1	0.0–0.2	−0.4a	0.3	4.0a	2.9	4.8a	4.4	−9.1a	5.1
2	0.2–0.4	−22.9b	19.4	−8.7ab	6.4	−1.9a	1.8	−16.0a	9.0
3	0.4–0.6	−9.5ab	8.1	−27.3c	20.1	−0.9a	0.8	−29.1a	16.4
4	0.6–0.8	−12.4ab	10.5	−18.1bc	13.3	−22.6b	20.9	−23.5a	13.2
5	0.8–1.0	−72.8c	61.7	−77.9d	57.3	−78.1c	72.1	−100.0b	56.3
	RI	−34.6 ± 7.7	100.0	−38.5 ± 1.0	100.0	−59.6 ± 8.4	100.0	−62.2 ± 3.3	100.0
		PGPR[b]							
		Comamonas acidovorans 30		*Comamonas acidovorans* 26		*Alcaligenes piechaudii*		*Agrobacterium* sp.	
		a	*b*	*a*	*b*	*a*	*b*	*a*	*b*
1	0.0–0.2	19.3a	21.8	13.6a	16.3	28.4c	34.9	20.4ab	14.6
2	0.2–0.4	1.0a	1.1	12.5a	15.0	16.3b	20.0	8.1ab	5.8
3	0.4–0.6	5.1a	5.8	14.8a	17.8	5.7ab	7.0	22.9a	16.5
4	0.6–0.8	22.1a	25.0	18.5a	22.2	12.1ab	14.9	−2.2b	1.6
5	0.8–1.0	−40.9b	46.3	−23.8b	28.6	−18.9d	23.2	−85.5c	61.5
	RS	43.9 ± 2.9	100.0	45.5 ± 5.0	100.0	48.0 ± 5.9	100.0	61.2 ± 5.6	100.0

[a]TLC plates were developed in ethyl acetate–chloroform–formic acid (55 : 35 : 10).
[b]*a*: The effect on root elongation (% of control), (−) signifies inhibition. *b*: Relative effect of each fraction of the bacterial eluate on root growth (%). Different letters represent significant differences ($p < 0.05$) among the different fractions in each bacterium; *a* also indicates not significant from the TLC control.

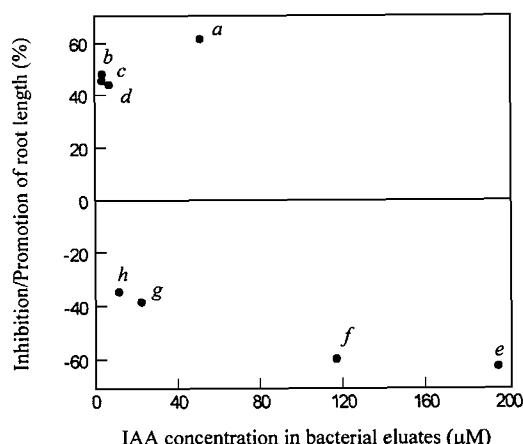

FIG. 2. The association between the concentration of IAA in bacterial eluates of either DRB or PGPR and root growth inhibition/promotion (letters represent different bacterial isolates listed in Table 1).

Separation and Characterization of IAA in Bacterial Eluates. Analysis of bactrial secretions by TLC with either of two different developing solutions (ethyl acetate–isopropanol–ammonium hydroxide or ethyl acetate–chloroform–formic acid), revealed that eight different bacterial isolates secreted a fraction with the same R_f value as authentic IAA. This fraction appeared at $R_f = 0.35$ when developed in ethyl acetate–isopropanol–ammonium hydroxide and at $R_f = 0.89$ when developed in ethyl acetate–chloroform–formic acid. The average concentration of IAA secreted from all DRB isolates was 4.7 times higher than that secreted by PGPR (cf. Table 1) and was significantly different ($p < 0.05$). A high negative correlation ($r = 0.66$) was found between the effect of the bacteria (DRB and PGPR) on root growth and the concentration of IAA in the bacterial eluates (Figure 2). However, the PGPR *Agrobacterium* sp. (Figure 2, *a*) secreted 51.2 μM of IAA and still induced root elongation (Table 2). In addition, the two DRB isolates, *Gluconobacter* sp. (Table 1, *g*) and *Pseudomonas putida* (Table 1, *h*) secreted relatively small amounts of IAA (22.3 and 11.7 μM) yet suppressed root elongation.

Application of different high concentrations of authentic IAA to roots of lettuce seedlings inhibited root growth. When applied at 1×10^{-5} μM, IAA promoted root growth by ca. 16%. However, much higher concentrations of IAA (16.4 μM) were found in the secretions of PGPR (Figure 2), suggesting that substances other than IAA may govern root growth promotion.

Inhibiting or Promoting Active Fractions in Bacterial Secretions. TLC fractionation revealed that the eluate derived from area 5 (R_f = 0.8–1.0) contained a

compound with an R_f value similar to authentic IAA (R_f = 0.89). When eventually isolated and amended to its original concentration in the bacterial eluate, this fraction inhibited root elongation, whether derived from DRB or PGPR (Table 2). The effect of each TLC fraction on lettuce root growth was compared to TLC control. It should be noted that when lettuce seeds were germinated on the eluate of a fraction of TLC control, root elongation was inhibited by ca. 12% compared with that of control seedlings grown in distilled water. The effect of the other fractions from eluates of DRB or PGPR on lettuce roots was variable. All fractions of the DRB eluates inhibited root elongation; but fractions of the eluates of PGPR, other than the IAA fraction, promoted root growth (Table 2). In nature, inhibitory potential of IAA may thus be masked. This was particularly true for *Alcaligenes piechaudii.* In this species, fractions other than IAA counted for a total stimulation of lettuce root growth of 62.5% compared to the control. Although the inhibitory effect of the IAA fraction from the other two PGPR strains, *Agrobacterium* sp. and *C. acidovorans* 30 (−85.5% and −40.9%, respectively), comprised 45–60% of the relative effect, the whole eluate from each of these isolates had a promoting effect (Table 2).

DISCUSSION

Loper and Schroth (1986) reported that the growth inhibiting effect of several deleterious rhizobacteria was related to IAA secretion. This is confirmed by our findings. Fractionation of eluates of PGPR revealed that IAA was secreted at lower levels (4.7 times less) compared to levels secreted by DRB (Table 1). A high negative association was found between the effect of the bacteria (DRB and PGPR) on root growth and the concentration of IAA, supporting the view that IAA concentration determines the extent of inhibition and promotion. Nevertheless, in a number of bacteria (Figure 2, *a*, *g*, *h*), the relationship between IAA concentration in bacterial eluates and their effect on lettuce root growth could not be predicted.

IAA released from PGPR inhibited root growth, while all other fractions different from IAA (except one) exhibited an effect similar to the control or promoted root growth (Table 2). This latter effect was specific to PGPR and was not observed in the eluate of any of the DRB strains. The concentrations of IAA in the eluates of PGPR were higher than the threshold of authentic IAA needed to promote root growth (Table 1). It is, therefore, suggested that, in PGPR, bacterial metabolites other than IAA are involved in root growth. The nature of these growth promoters is as yet unknown. It has been suggested that succinic and lactic acids eluated from *Pseudomonas putida* are involved in the promotion of root elongation (Yoshikawa et al., 1993). ACC deaminase also has been suggested as accounting for the promotion of *Brassica campestris* by *P. putida* (Glick et

al., 1994). When root growth is mediated by IAA from microflora, various environmental factors e.g., dilution, leaching, and oxidation, may reduce inhibitory levels of IAA to concentrations that are effective in stimulating growth. Thus, it is possible that bacteria may stimulate root growth by several metabolites including low concentrations of IAA.

Isolation and purification of fractions recovered from the eluates of DRB revealed that IAA accounted for ca. 60–70% of the total inhibitory effect (Table 2). All other fractions in the eluate of DRB also inhibited root growth of lettuce (Table 2). Interestingly, when IAA in the eluate was separated by TLC and reapplied to lettuce seedlings (at a concentration identical to that in the original eluate), the inhibitory effect increased by 1.3–2.1 times (Table 2) compared with the effect of the unfractionated bacterial eluate. This is exemplified by the eluate of *Micrococcus luteus*, which inhibited root elongation by 62% compared with control seedlings (Figure 1). However, after fractionation, the fraction at R_f = 0.89, amended to its original concentration in the eluate, completely arrested root elongation (100%). It is possible that an antagonistic effect occurs among the different fractions and/or with IAA as a major inhibitor.

For synthesizing IAA, rhizobacteria require L-tryptophan supplements; these are obtained by bacteria from living root exudates or from disintegrating plants (Patten and Glick, 1996). L-Tryptophan has been found in the exudates of roots of seedlings of *Avena sativa* (0.25 mM) in a concentration that inhibits root growth of lettuce seedlings by ca. 50% (Kato-Noguchi et al., 1994). In our growing medium (MYA), the intrinsic concentration of L-tryptophan was lower (ca. 0.16 mM) (Rechcigl, 1978). We suggest that in the rhizoplane, L-tryptophan is released at levels that inhibit root growth directly and also indirectly through amplifying the production of IAA by deleterious root-colonizing bacteria. Since phytotoxic or growth-promoting compounds may also increase root exudation (Meharg and Killham, 1995), by amplifying IAA secretion, bacteria may benefit from elevated amounts of nutrients.

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ANNOUNCEMENT

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ALLELOPATHIC SUPPRESSION OF *Pseudomonas solanacearum* INFECTION OF TOMATO (*Lycopersicon esculentum*) IN A TOMATO-CHINESE CHIVE (*Allium tuberosum*) INTERCROPPING SYSTEM

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Abstract—Tomato plants were grown alone or intercropped with Chinese chive plants, with or without the inoculation of *Pseudomonas solanacearum*. Chinese chive plants had no detrimental effects on the growth of tomato plants but significantly delayed and suppressed the occurrence of bacterial wilt of tomato. *P. solanacearum* population decreased faster in the soil grown with tomato alone than that in the soil grown with both tomato and Chinese chive. However, *P. solanacearum* population in bare soil was higher than that grown with Chinese chive. Root exudates of Chinese chive collected with a continuously trapping system were inhibitory to multiplication of *P. solanacearum*.

Key Words—Allelopathy, *Allium tuberosum*, antibacterial activity, biological control, intercropping, *Lycopersicon esculentum* Mill, *Pseudomonas solanacearum* E. F. Smith, root exudates, soil-borne diseases.

INTRODUCTION

Soil-borne disease has been a serious problem in the production of some vegetable and cut-flower crops. Although use of resistant cultivars, grafting, and soil fumigation are practices adopted widely, soil-borne diseases still cause great loss in many countries. As a consequence of public concern for environmental damage caused by continued use of conventional synthetic agrochemicals, sustainable agriculture has gained importance in both developed and developing countries (Rice, 1995).

There is increasing evidence that allelopathy plays an important role in agri-

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cultural and ecological systems. Much work has been done to investigate the detrimental effects of allelopathy but little on the beneficial aspects of allelopathy in agricultural systems (Putnam, 1986). It is known that many plants release allelochemicals by exudation, decomposition, leaching, and vaporization. Allelochemicals mostly in the rhizosphere may be toxic or stimulatory to the releaser, to other plant species, and to soil microorganisms (Grodzinsky, 1992; Kuniyasu, 1989; Papavizas, 1966; Rice, 1995; Yu and Matsui, 1994). Therefore, allelopathy could be managed to improve crop productivity or to decrease losses caused by weeds or other pests. Although the concept of biological control of soil-borne disease by allelopathic approach has been recognized (Fujii, 1989; Patrick, 1986; Rice, 1995), there is need for additional experimental support of the process.

We are interested in an allelopathic approach to biological control of soil-borne diseases of vegetable crops. In a previous study, we reported the suppressive effects of sugi (*Cryptomeria japonica* D.) bark on some root infection diseases of tomato (Yu et al., 1997). The objective of this report is to examine the effects of allelopathic control of tomato bacterial wilt caused by *Pseudomonas solanacearum* Smith in a Chinese chive–tomato intercropping system.

METHODS AND MATERIALS

Pathogen. Pseudomonas solanacearum Smith Ps5 used in this study was isolated from a tomato field in Hangzhou, Zhejiang, China.

Plants. Tomato (*Lycopersicon esculentum* Mill cv. Zaofen No. 2) and Chinese chive (*Allium tuberosum* cv. Hangzhou snow-Chinese chive) were used.

Experiment 1: *Effects of Intercropping with Chinese Chive on Growth of Tomato Plants.* The experiment carried out in the spring of 1997 consisted of three treatments: A tomato plant monocropping, a tomato–Chinese chive intercropping, and a Chinese chive monocropping. Sixteen Chinese chive seedlings were transplanted in three lines in a box (40 × 60 × 10 cm) filled with sandy soil. Six tomato seedlings at the four-leaf stage were transplanted one month after starting the Chinese chive. Each box received 0.2–1.5 liters of Enshi nutrient solution (Yu and Matsui, 1994) daily, depending on the weather and plant growth stage. The experiment lasted for 60 days. Leaf number and plant height of the tomato plants were recorded weekly. Shoots and roots were harvested, dried at 80°C, and weighed. Each treatment consisted of two boxes and was conducted in triplicate in a randomized block design.

Experiment 2: *Effect of Intercropping Chinese Chive with Tomato on Tomato Bacterial Wilt.* At the end of experiment 1, one box from tomato monocropping and one box from tomato–Chinese chive intercropping, were transplanted with tomato seedlings. The experiment consisted of a tomato monocropping as control and a tomato–Chinese chive intercropping. The experiment was

conducted in a manner similar to experiment 1, except that each box was inoculated with 1 liter of *P. solanacearum* suspension two weeks after transplanting of tomato seedlings. *P. solanacearum* suspension was prepared from stem segments of diseased tomato plants and the concentration of the pathogen was adjusted to 10^7 cells/ml (Yu et al., 1997). Tomato plants that wilted were recorded daily, and counts of pathogens in the soils were conducted every four days. The experiment ended two weeks after the inoculation. Each treatment, consisting of one box, was replicated three times in a randomized block design.

Experiment 3: *Effects of Chinese Chive as Preceding Crops on Tomato Bacterial Wilt.* At the end of the experiment 1 in 1997, a box from tomato monocropping (bare soil without plants) and a box from tomato–Chinese chive intercropping (with Chinese chive plants only) were each inoculated with 1 liter of *P. solanacearum* suspension at a concentration of 10^7 cells/ml. The boxes were kept moist by irrigating with water or a one-fifth-strength Enshi nutrient solution, respectively, for bare soil and Chinese-chive-grown soil. Chinese chive leaves were harvested two times during the experiment. The soil was sampled every two months during the experiment for the counting of pathogen population. Tomato seedlings were transplanted into the boxes one year after (June 10, 1998) the inoculation of *P. solanacearum*. Tomato seedlings were irrigated with half-strength Enshi nutrient solution daily and plants that wilted were recorded. The experiment ended three weeks after the transplanting of tomato seedlings. Each treatment consisted of one box and was replicated three times in a randomized block design.

Collection of Root Exudates of Chinese Chive. Chinese chive plants from Chinese monocropping in experiment 1 were used for the collection of root exudates of Chinese chive. A continuous trapping system for root exudates was used (Tang and Young, 1982). The procedure was similar to that for the collection of cucumber root exudates (Yu and Matsui, 1994). Ten Chinese chive plants were grown in each pot and root exudates were collected on an Amberlite XAD-4 resin column for 30 days, eluted with methanol, and evaporated to dryness at reduced pressure. Two hundred fifty milligrams of root exudates were obtained.

Effects of Root Exudates of Chinese Chive on P. solanacearum. The procedure was the same as that for the assay of antibacterial activity of sugi bark extracts (Yu et al., 1997). *P. solanacearum* was inoculated in a liquid potato–sugar (PS) medium with or without the addition of root exudates. The dried root exudate was first dissolved in ethanol and the final concentration in the PS solution was 100 mg/liter.

Counting of Pathogen Population in Soil and PS Medium. Ten milliliters of soil or 1 ml of suspension was collected from each box or flask, respectively, and added to a flask with 100 ml of sterile deionized distilled water. After vigorous shaking, serially diluted aliquots were plated on an improved Drigalsuki medium (Soil-borne Diseases Countermeasure Committee of Japan, 1974). After incubat-

ing for three days at 28°C, colonies appearing on the plates were counted, and the number of viable cells in the soil or the suspension at the time of sampling was estimated from the count (Yu et al., 1997).

RESULTS

Effect of Intercropping with Chinese Chive on Growth of Tomato Plants. There were no significant differences in the height, leaf number, and dry matter production between tomato plants grown alone and tomato plants intercropped with Chinese chive. No unusual symptoms, such as nutrient deficiency, were observed in tomato plants intercropped with Chinese chive plants throughout the experiment. However, the growth of Chinese chive intercropped with tomato was better than when grown alone. For example, plant height was increased by 10.8% as compared to that grown alone ($P < 0.05$, data not shown).

Effect of Intercropping with Chinese Chive on Tomato Bacterial Wilt. The pathogen population in soil grown with tomato alone decreased and reached 10^5 cells/ml by the end of the experiment, 20 days after inoculation. The pathogen population in the soil grown with tomato and Chinese chive was unexpectedly 10 times higher than that of the control soil and was 10^6 cells/ml by the end of the experiment (Figure 1). Figure 2 shows that when pathogens were inoculated two weeks after the transplanting of tomato, plants grown alone began to wilt five days after inoculation and wilting reached 100% by 11 days. Wilting was greatly suppressed in tomato plants intercropped with Chinese chive (maximum

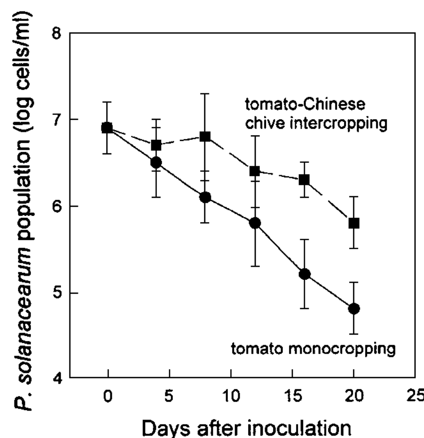

FIG. 1. *P. solanacearum* populations in soils grown with tomato plants alone (●) and those grown with both tomato and Chinese chive plants (■) in experiment 2. Data are the mean values of three measurements with standard errors shown by the vertical bars.

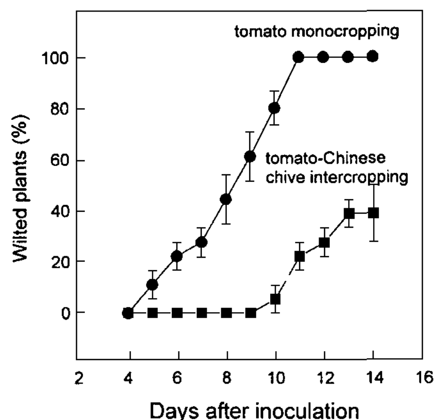

FIG. 2. Development over time of bacterial wilt of tomato plants grown alone (●) or intercropped with Chinese chive (■) in experiment 2. Data are the mean values of three measurements with standard errors shown by the vertical bars.

wilting was about 40%, Figure 2), beginning only 10 days after inoculation, and the wilting percentage also was lower than that of the control plants.

In experiment 3, pathogens were inoculated on June 15, 1997, into bare soil and into soil grown with Chinese chive. Both of these soils were used to grow tomatoes from May 1997 throughout June 9, 1997. The pathogen population in the bare soil was constant during the experiment, while pathogen population in the soil grown with Chinese chive decreased (Figure 3). When tomato seedlings were transplanted into the bare soil in the succeeding year (June 10, 1998), all the plants died 20 days after transplanting. The incidence of bacterial wilt in tomato seedlings transplanted into the box with Chinese chive, however, was greatly suppressed, starting only on day 12 after inoculation (seven days later than in the control plants), and the wilting percentage was only 22.2% at the end of experiment when all control plants had died (Figure 4).

Effects of Root Exudates of Chinese Chive on Multiplication of P. solanacearum. *P. solanacearum* multiplied very fast in a PS medium. The cell density could reach 10^{12} cells/ml in 36 hr. Root exudates of Chinese chive at a concentration of 100 mg/liter inhibited the growth of *P. solanacearum*, and the cell density was only 1% of the control 30 hr after inoculation (Figure 5).

DISCUSSION

Intercropping is widely adopted in developing countries practicing intensive agriculture, and it also has received attention in developed countries as an

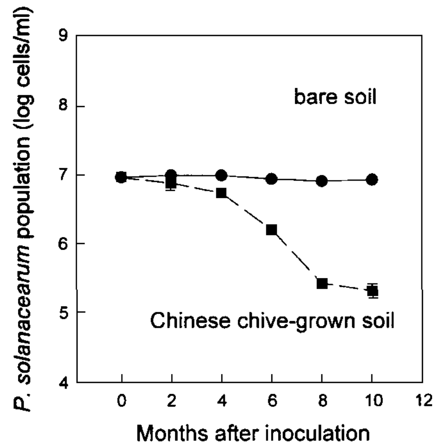

FIG. 3. Population dynamics of *P. solanacearum* in bare soils (●) and in those grown with Chinese chive plants (■) in experiment 3. Data are the mean values of three measurements with standard errors shown by the vertical bars.

approach to sustainable horticulture. The importance of allelopathy in cropping systems has been recognized in recent years (Theunissen, 1997). Our results showed that there were no allelopathic effects of Chinese chive plant on the growth of tomato plant based on comparison of plant height, leaf number, and

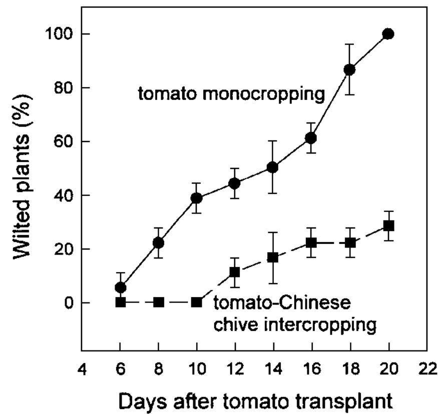

FIG. 4. Development over time of bacterial wilt of tomato plants grown alone (●) or intercropped with Chinese chive (■) in experiment 3. Data are the mean values of three measurements with standard errors shown by the vertical bars.

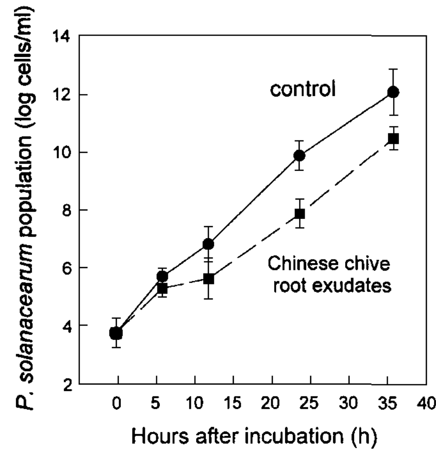

FIG. 5. Multiplication of *P. solanacearum* in liquid media with (■) or without root exudates of Chinese chive (●). Data are the mean values of three measurements with standard errors shown by the vertical bars.

dry matter production between tomato plants grown alone and tomato plants intercropped with Chinese chive (data not shown). The growth of Chinese chive intercropped with tomato was much better than Chinese chive grown alone, which we attributed to the shading of Chinese chive by the tomatoes. High temperatures together with strong sunlight during the summer are not suitable for the growth of Chinese chive (Lee, 1988). These results showed that intercropping tomato with Chinese chive is compatible and may have beneficial effects on the growth of Chinese chive.

It has been noted that allelopathy can be managed to control plant pests and diseases (Fujii, 1989; Patrick, 1986; Putnam, 1986; Rice, 1995). Some studies also have shown the possibility of biological control of insect pests and nematodes in intercropping systems by allelopathy (Ito et al., 1994; Theunissen, 1997). Compounds such as glycosides and α-terthienyl were isolated and identified as allelochemical (Rohde, 1960; Tang et al., 1987). Yamagishi et al. (1986) found that the incidence of clubroot disease (*Plasmodiophora brassicae* Worn.) in turnip was suppressed together with a drastic decrease in the density of resting spores in soils when resistant kale and turnip strains were grown as preceding crops in the spring.

In agreement with these studies, our study showed that intercropping with Chinese chive greatly inhibited the occurrence of bacterial wilt in tomato. This could be observed in both the onset time of plant wilting and the percentage of wilted plants. This suppressive effect was further ascertained in experiment 3 in

which *P. solanacearum* was inoculated one year prior to tomato seedling transplant, and the effect was more apparent than that in experiment 2. Root exudates of Chinese chive were inhibitory to, but were not able to kill, *P. solanacearum*. Root exudates of Chinese chive may prevent *P. solanacearum* from infecting tomato plants. Lower populations of *P. solanacearum* in the soil and lower percentages of plant wilting were observed in intercropped soil than in monocropped soil in experiment 2. It is not difficult to understand the higher levels of pathogen populations in bare soil than those in soil grown with Chinese chive in experiment 3 because of inhibition of *P. solanacearum* by root exudate of Chinese chive. When tomato plants were grown in the Chinese chive-grown soil with lower pathogen population, fewer plants wilted. When tomato plants were present, a portion of the pathogen population infected the plants. Accordingly, the pathogen population decreased more rapidly in soils with tomato plants (Figure 1) than in soil without tomato plants. Chinese chive plants also contain some sulfur compounds such as *S*-methyl-2-propenethiosulfinate, which showed strong activity in inhibiting the pathogen responsible for carnation bacterial wilt (Takenaka et al., 1991). Therefore, it also may be possible to control other soil-borne disease by growing Chinese chive with other crops.

Our results indicated that soil-borne disease controlled by allelopathic approaches in cropping systems could result in producing higher quality and safer products at lower cost.

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MOTH SCALE-DERIVED KAIROMONES USED BY EGG–LARVAL PARASITOID *Ascogaster quadridentata* TO LOCATE EGGS OF ITS HOST, *Cydia pomonella*

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Abstract—We determined that location of host (*Cydia pomonella*) eggs by *Ascogaster quadridentata* is mediated by kairomones, investigated potential sources of the kairomones and identified a blend of kairomones from the source that was attractive to *A. quadridentata*. In Y-tube olfactometer bioassays, female *A. quadridentata* were attracted to Porapak Q-collected volatiles from female *C. pomonella* scales and eggs, but not to *C. pomonella* sex pheromone. Scales of *C. pomonella* were also attractive to male *A. quadridentata*. Coupled gas chromatographic–electroantennographic detection analysis of scale volatile extracts revealed numerous compounds that elicited responses from male or female *A. quadridentata* antennae, including heptanal, octanal, nonanal, decanal, undecan-2-one, dodecanal, pentadecan-2-one, (*Z*)-6-pentadecen-2-one, (*Z*)-9-hexadecenal, (*Z*)-6-heptadecen-2-one, and 3,7,11-trimethyl-2*E*,6*E*,10-dodecatrien-1-ol acetate. A synthetic blend of these compounds at quantities and ratios equivalent to Porapak Q scale volatile extract was attractive to female *A. quadridentata* in a Y-tube olfactometer bioassay.

Key Words—Kairomone, parasitoid, *Ascogaster quadridentata*, *Cydia pomonella*, host location, foraging behavior, courtship displays, wing fanning, heptanal, octanal, nonanal, decanal, undecan-2-one, dodecanal, pentadecan-2-

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one, (*Z*)-6-pentadecen-2-one, (*Z*)-9-hexadecenal, (*Z*)-6-heptadecen-2-one, and 3,7,11-trimethyl-2*E*,6*E*,10-dodecatrien-1-ol acetate.

INTRODUCTION

Parasitoids use diverse chemical cues to locate their hosts. Typically, they exploit cues that reliably indicate the presence of a suitable host (Vet et al., 1995). These cues may originate from the habitat of the host or from the host itself (Shaw and Huddleston, 1991; Godfray, 1994). As *Ascogaster quadridentata* Wesmael (Hymenoptera: Braconidae) is a solitary egg–larval koinobiont endoparasitoid that attacks primarily Tortricidae eggs, this parasitoid might utilize sex pheromone components of the host moth (Lewis et al., 1982), volatiles from scales deposited by the female moth during oviposition (Lewis et al., 1972; Chiri and Legner, 1986; Kainoh et al., 1990), and/or volatiles from the host egg itself (Vinson, 1975) to locate oviposition sites.

Among the large number of microlepidoptera that *A. quadridentata* will parasitize, the codling moth, *Cydia pomonella* L. (Lepidoptera: Tortricidae), is the most economically important as it is a severe pest of apples on a worldwide scale (Clausen, 1978). The development of resistance in *C. pomonella* to heterocyclic organophosphates, such as Guthion (Brown, 1993), the deregistration of many insecticides, and the detrimental impact of insecticides on beneficial insects and the environment (Putman, 1963; Dolstad, 1985; Brown, 1993; Riddick and Mills, 1994) have generated great interest in alternative controls for *C. pomonella*. *A. quadridentata* shows promise for reducing *C. pomonella* populations and therefore could serve as an important component in an integrated management system for *C. pomonella* when organophosphates are replaced by more benign controls (Brown, 1993). Understanding the chemical ecology of *A. quadridentata* may enhance this effort. Thus, the focus of this paper was to identify kairomones derived from the codling moth and to demonstrate attraction of parasitoids to a synthetic kairomone blend.

Research objectives were to: (1) investigate whether host location by *A. quadridentata* is mediated by kairomones; (2) determine the source(s) of kairomones; and (3) identify and bioassay candidate kairomones for their attractiveness to *A. quadridentata*.

METHODS AND MATERIALS

Acquisition of Volatile Stimuli from Potential Kairomonal Sources. Volatile test stimuli consisted of the following: (1) synthetic *C. pomonella* pheromone, (*E*,*E*)-8,10-dodecadien-1-ol (codlemone), (2) pheromone gland extract of female *C. pomonella*; (3) volatiles from *C. pomonella* eggs; and (4) volatiles from body and wing scales of female *C. pomonella*.

Synthetic codlemone (98% chemical purity) was obtained from Shin-etsu (Fine Chemical Department, Chemical Co. Ltd., Tokyo). Pheromone gland extracts of female *C. pomonella* were obtained by removing abdominal tips with pheromone glands from 108 calling virgin female *C. pomonella*, 48–72 hr old, and extracting them for ca. 10 min in HPLC-grade hexane. The supernatant was withdrawn and stored at ca. $-10°C$ until use. A 1-μl aliquot of this extract contained 1 female equivalent of pheromone gland extract (1 FE PGE).

Egg volatiles were collected from ca. 5000 eggs that had been oviposited by female *C. pomonella* on a wax-paper sheet (1950 cm^2). The egg source was placed in a cylindrical Pyrex glass chamber (ca. 155 mm ID × 280 mm in height) and was aerated for 72 hr. A water aspirator drew charcoal-filtered air at 2 liters/min through the chamber and a connected glass column (14 cm × 1.3 mm OD) filled with Porapak Q. Volatiles were eluted from the Porapak Q with 3 ml of redistilled pentane. One microliter of extract was equivalent to ca. 120 egg hour equivalents (120 EHE; i.e., volatiles released from 120 eggs of *C. pomonella* during 1 hr).

To collect volatiles from body and wing scales of female *C. pomonella*, 500 chilled female moths were placed into the upper half of two Petri dishes (8.5 cm diam.) separated by a fine wire mesh (1 mm^2). The insects were then shaken and displaced scales collected in the bottom Petri dish were transferred to a cylindrical Pyrex glass aeration tubing (2.5 cm diam. × 18.5 cm long). A water aspirator drew charcoal-filtered air through the tubing and a volatile trap containing Porapak Q (see above) at a rate of 1.2 liters/min for 142 hr. Captured volatiles were eluted from the Porapak Q with 3 ml of redistilled pentane. A 1-μl aliquot contained the equivalent of volatiles emitted for 1 hr from the scales displaced from 24 female *C. pomonella* [24 female scale hr equivalents (SHE)].

Testing of Potential Kairomone Sources. Potential kairomone sources were tested for attractiveness to *A. quadridentata* in Y-tube olfactometer experiments. Responses of walking or flying *A. quadridentata* to odor sources were assessed at 25–27°C and 50–70% relative humidity in vertical Y-shaped Pyrex glass olfactometers [stem: 20 cm long × 2.5 cm ID; side arms at 120°: 18 cm long; entrance hole for parasitoids (4 mm diam.) 5 cm above the rim of the stem]. Bioassays were conducted between hrs 2 and 12 of the insects' photophase (16L : 8D, photoregime). Because *A. quadridentata* is positively phototactic, a single light source composed of tubes of fluorescent "daylight" (F40D H568, Osram Sylvania Ltd.) and "wide-spectrum grow light" (F40GRO WS6 H568, Osram Sylvania Ltd.) at a 1 : 1 ratio (Shields, 1989) was centered above the vertical olfactometer. Radiometric irradiance at the top and base of the olfactometer was 8.0 W/m^2 and 3.8 W/m^2, respectively (Radiometer model IL1400A, International Light Inc.). Visual cues were standardized by enclosing the olfactometer on three sides with white poster board. Treatment and control odor sources were micropipetted onto Whatman No. 1 filter paper (4.25 cm) placed near the orifice of

each side arm. For each replicate, a clean (Sparkleen-washed and oven-dried) Y-tube, insect, and filter paper were used, with test stimuli randomly assigned to side arms. Air drawn through the apparatus at 2.4–3.3 liters/min with a water aspirator carried volatiles from odor sources through the stem of the Y-tube. Thirty seconds after placement of stimuli, a parasitoid was released through the entrance hole of the olfactometer. Parasitoids that reached a filter paper containing an odor source within 15 min were classed as responders; all others were classed as nonresponders and were not included in statistical analyses.

Numbers of parasitoids responding to stimuli in olfactometer bioassays (>85%) (Tables 1 and 2) were analyzed with the χ^2 goodness-of-fit test using Yates' correction for continuity ($\alpha = 0.05$) to determine whether observed frequencies deviated significantly from expected frequencies, under the null hypothesis that sampled *A. quadridentata* did not prefer either treatment or control odors (Zar, 1996).

Pheromone gland extract (1 FE PGE) was tested with females (experiment 1) and males (experiment 10). Synthetic codlemone at 10, 1, and 0.1 ng was tested with females (experiments 1–4) and with males (experiments 11–13). Volatiles from eggs of *C. pomonella* at 120 EHE were tested with unprimed and primed female *A. quadridentata* (experiments 5 and 6). Priming was attained by exposing female parasitoids to *C. pomonella* eggs and allowing them to oviposit once prior to experiments, while unprimed females had no previous contact with *C. pomonella* eggs prior to experiments. Priming lowers the behavioral response threshold to stimuli (Kaiser and Cardé, 1992; Harris and Foster, 1995) and ensures that females not interested in host location are excluded from experiments. Egg volatiles at 120 EHE and 1200 EHE also were tested with male *A. quadridentata* (experiments 14 and 15). Volatiles from body and wing scales of female *C. pomonella* were tested at 24 SHE with female *A. quadridentata* (experiment 7) and at 240 SHE with male *A. quadridentata* (experiment 16). Controls consisted of the same solvent, either hexane or pentane, at the equivalent volume (0.5–10 μl) used for the corresponding treatments.

Identification of Candidate Kairomones. Aliquots of 1 FE PGE, 120 EHE, and 24 SHE of volatile extracts were subjected to analyses by coupled gas chromatographic–electroantennographic detection (GC-EAD) (Arn et al., 1975), employing a Hewlett Packard (HP) 5890A gas chromatograph equipped with a fused silica column (30 m × 0.25 or 0.32 mm ID) coated with DB-5, DB-23, or DB-210 (J & W Scientific, Folsom, California). For GC-EAD recordings, an antenna was gently pulled from an insect's head; the distal segment was removed, and it was then suspended between glass capillary electrodes filled with insect Ringer's solution (6.5 g/l NaCl, 1.4 g/l KCl, 0.12 g/l $CaCl_2$, 0.1 g/l $NaHCO_3$, 0.01 g/l Na_2HPO_4, in 1 l of distilled H_2O). EAD-active compounds were identified by using full-scan electron-impact (EI) and chemical-ionization (isobutane) (CI) mass spectra (MS) obtained from a Varian Saturn II Ion Trap

GC-MS and an HP 5985B GC-MS, respectively (each fitted with the DB-5 or DB-210 column referred to above), and by retention index calculations (Dool and Kratz, 1963). Structural assignments of EAD-active scale volatiles were confirmed by comparative GC, GC-MS, and GC-EAD analyses of insect-produced compounds and authentic standards.

Purity and source of synthetic standards are as follows: (1) heptanal, >95% purity, obtained by oxidizing heptanol (from Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada); (2) octanal, >95% purity (from Sigma-Aldrich Canada); (3) nonanal, >95% purity, obtained by oxidizing nonanol (from Sigma-Aldrich Canada); (4) decanal, 99% purity (from Sigma-Aldrich Canada); (5) undecan-2-one, >99% purity (from Fluka Chemika-Biochemika, Buchs, Switzerland); (6) dodecanal, 92% purity (from Sigma-Aldrich Canada); (7) (Z)-6-pentadecen-2-one, 95% purity (synthesized by the authors); (8) pentadecan-2-one, >97% purity (synthesized by G. G. S. King, Simon Fraser University); (9) (Z)-9-hexadecenal, 95% purity (from Sigma-Aldrich Canada); (10) (*E,E*)-farnesyl acetate (Adams, 1989), 95% purity (from Aldrich Chemical Co., Milwaukee, Wisconsin); and (11) (Z)-6-heptadecen-2-one, >91% purity (synthesized by the authors).

Synthetic equivalents of the 11 EAD-active compounds identified from scale volatile extract of female *C. pomonella* were combined in quantities and ratios as found in the scale volatile extract. This synthetic blend, versus hexane controls, was tested at 24 SHE (experiment 8) and 240 SHE (experiment 9) with female *A. quadridentata* in Y-tube olfactometer bioassays.

RESULTS

Behavioral Evidence for Kairomone-Mediated Host Location. The results from experiments 1–9 are presented in Table 1. In olfactometer bioassays, pheromone gland extracts of female *C. pomonella* failed to attract female *A. quadridentata* (experiment 1). Codlemone at doses of 10, 1, or 0.1 ng was no more attractive to female *A. quadridentata* than the hexane control (experiments 2–4).

In contrast, volatiles from eggs of *C. pomonella* were attractive to primed female parasitoids (experiment 6) but unattractive to unprimed female parasitoids (experiment 5). In the presence of egg volatiles, female parasitoids exhibited behaviors such as wing fanning and occasional antennal tapping (antennae contact the substrate in a tapping motion). Volatiles from scales of female *C. pomonella* (experiment 7) were attractive to female *A. quadridentata*, elicited wing fanning and regularly induced antennal tapping.

The results from experiments 10–16 are presented in Table 2. Neither pheromonal extract of female *C. pomonella* (experiment 10), synthetic codlemone (experiments 11–13), nor egg volatiles (experiments 14 and 15) were

TABLE 1. STIMULI TESTED AND RESULTS OBTAINED IN Y-TUBE OLFACTOMETER EXPERIMENTS, BIOASSAYING FEMALE *A. quadridentata*

Exp.	Treatment[a]	Parasitoid status	Tested (*N*)	Responding to either stimulus (*N*)	Responders choosing treatment (%)	*P*[b]
1	1 FE PGE in hexane	Mated Unprimed	28	27	52	1
2	10 ng codlemone in hexane	Virgin Unprimed	13	12	58	0.7728
3	1 ng codlemone in hexane	Virgin Unprimed	11	10	60	0.7518
4	0.1 ng codlemone in hexane	Mated Unprimed	20	20	45	0.8231
5	120 EHE in pentane	Mated Unprimed	20	18	72	0.0990
6	120 EHE in pentane	Mated Primed	20	19	84	0.0059**
7	24 SHE in pentane	Mated Primed	16	15	80	0.0389*
8	Synthetic scale volatiles (24 SHE) in hexane	Virgin Primed	26	23	74	0.0370*
9	Synthetic scale volatiles (240 SHE) in hexane	Virgin Primed	20	20	60	0.5023

[a]Abbreviations and explanations: 1 FE PGE = female *C. pomonella* equivalent of pheromone gland extract (= volatiles extracted from the pheromone gland of 1 female *C. pomonella*). 1 EHE = egg hour equivalent (= volatiles released from 1 egg of *C. pomonella* during 1 hr). 1 SHE = female *C. pomonella* scale hour equivalent [= volatiles released from body and wing scales (displaced from 1 female *C. pomonella*) during 1 hr}. Codlemone = (*E,E*)-8,10-dodecadien-1-ol, sex pheromone of female *C. pomonella*. Controls consisted of the same solvent, either hexane or pentane, at the equivalent volume (0.5–10 μl) used for the corresponding treatments.

[b]χ^2 test with Yates' correction for continuity, treatment versus control; asterisks indicate a significant response to a particular treatment; $^*P < 0.05$; $^{**}P < 0.01$.

attractive to male *A. quadridentata* in Y-tube olfactometers. However, volatiles from female *C. pomonella* scales at a high dose did attract male *A. quadridentata* (experiment 16). Males wing-fanned occasionally in the presence of codlemone, egg, or scale volatiles.

Identification of Candidate Kairomones. In coupled GC-EAD recordings, codlemone elicited only a very weak response from female *A. quadridentata* antennae, while other components of female *C. pomonella* pheromone gland extract were not EAD-active (Figure 1). In contrast, male *A. quadridentata* antennae responded strongly to codlemone (Figure 1).

In GC-EAD recordings with volatiles from scales of *C. pomonella* (Figure 2), numerous volatiles elicited antennal responses from both female and male *A.*

TABLE 2. STIMULI TESTED AND RESULTS OBTAINED IN Y-TUBE OLFACTOMETER EXPERIMENTS, BIOASSAYING MALE *A. quadridentata*

Exp.	Treatment[a]	Tested (*N*)	Responding to either stimulus (*N*)	Responders choosing treatment (%)	*P*[b]
10	1 FE PGE in hexane	12	12	42	0.7728
11	10 ng codlemone in hexane	30	30	53	0.8551
12	1 ng codlemone in hexane	33	32	62	0.2159
13	0.1 ng codlemone in hexane	20	20	55	0.8231
14	120 EHE in pentane	20	18	39	0.4795
15	1200 EHE in pentane	18	17	41	0.6276
16	240 SHE in pentane	18	17	82	0.0153*

[a]Abbreviations and explanations are as in Table 1. Controls consisted of the same solvent, either hexane or pentane, at the equivalent volume (1–10 μl) used for the corresponding treatments.
[b]χ^2 test with Yates' correction for continuity, treatment versus control; asterisks indicate a significant response to a particular treatment; $^*P < 0.05$.

quadridentata. EAD-active compounds were further subjected to GC-MS, and their identification (Figure 2) confirmed by comparative GC, GC-MS, and GC-EAD analyses of insect-produced and authentic standards.

Synthetic scale volatiles were attractive to female *A. quadridentata* at 24

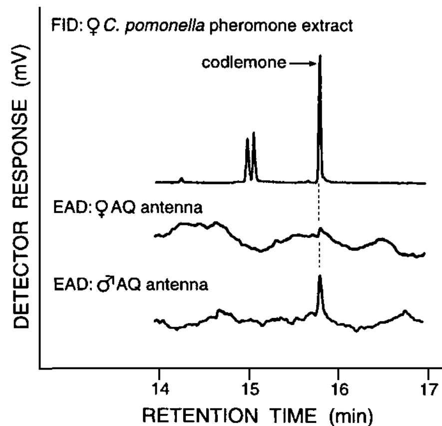

FIG. 1. Flame ionization detector (FID) and electroantennographic detector (EAD) responses to aliquots of 1 female equivalent of pheromone gland extract from female *C. pomonella*. Chromatography: Hewlett Packard 5890A equipped with a DB-5-coated column (30 m × 0.25 mm ID); linear flow velocity of carrier gas: 35 cm/sec; injector and FID detector temperature: 240°C; temperature program: 1 min at 50°C, 10°C/min to 240°C.

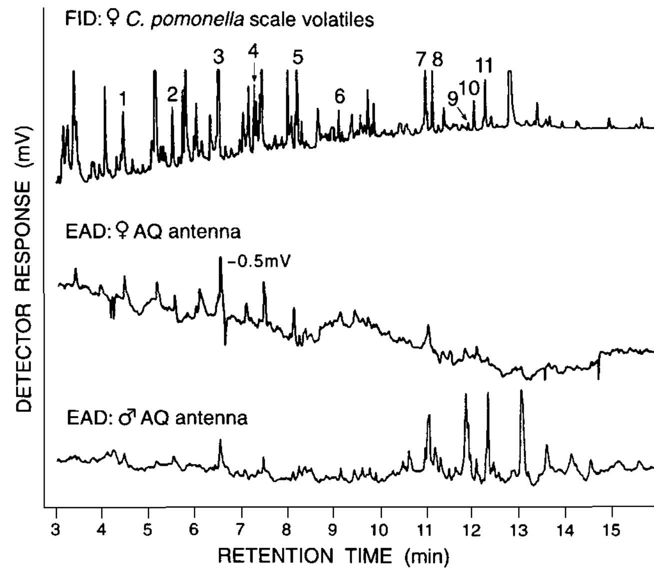

FIG. 2. Flame ionization detector (FID) and electroantennographic detector (EAD) responses to aliquots of 24 female scale hr equivalents (SHE) of volatiles from *C. pomonella*. Chromatography: Hewlett Packard 5890A equipped with a DB-5-coated column (30 m × 0.25 mm ID); linear flow velocity of carrier gas: 35 cm/sec; injector and FID detector temperature: 240°C; temperature program: 1 min at 50°C, 20°C/min to 280°C. Name and quantity of EAD-active compounds 1–11 are as follows: (1) heptanal: 1.5 ng, (2) octanal: 1.3 ng, (3) nonanal: 4.5 ng, (4) decanal: 4 ng, (5) undecan-2-one: 2.8 ng, (6) dodecanal: 0.6 ng, (7) (*Z*)-6-pentadecen-2-one: 1.7 ng, (8) pentadecan-2-one: 1.3 ng, (9) (*Z*)-9-hexadecenal: 0.1 ng, (10) (*E,E*)-farnesyl acetate: 0.7 ng, and (11) (*Z*)-6-heptadecen-2-one: 1 ng.

SHE (experiment 8) but not at 240 SHE (experiment 9), although both concentrations elicited wing fanning and regularly induced antennal tapping during bioassays. The high concentration appeared to induce general searching behavior, while the low concentration appeared to induce a directional response.

DISCUSSION

Location of host eggs by egg–larval parasitoids, such as *A. quadridentata*, may be mediated by pheromones from female moths, volatiles from scales deposited by female moths during oviposition, and/or by volatiles from the host egg itself. Moth pheromones retained by foliage on which calling and mating have occurred serve as kairomones in several parasitoids (e.g., Lewis

et al., 1982; Noldus and Lenteren, 1985; Noldus, 1989). Insignificant antennal responses from female *A. quadridentata* to synthetic codlemone (Figure 1), and no behavioral response to codlemone (experiments 2–4) or hexane extract of *C. pomonella* pheromone glands (experiment 1), provided strong evidence that the sex pheromone of female *C. pomonella*, on its own, is not a kairomone for foraging female *A. quadridentata*. Because mated female *C. pomonella* widely distribute their eggs, rarely depositing two or more eggs on the same fruit or leaf (Borden, 1931), pheromone adhering to the mating site may not be a reliable semiochemical for foraging female *A. quadridentata*.

Volatiles from eggs of *C. pomonella* attracted female parasitoids (experiment 6). Egg volatiles also induced wing fanning by male and female *A. quadridentata*. While wing fanning in response to female pheromone is part of many hymenopteran male parasitoid courtship displays (Assem, 1986), it has not been reported for male or female parasitoids in response to host kairomones. Vinson (1972) hypothesized that male wing fanning causes air movements that help parasitoids orient to the pheromone source. The behavior observed for female *A. quadridentata* supports this hypothesis, as females increased their frequency of wing fanning in response to lower doses of attractive kairomones. Following semiochemical-mediated location of host habitat, female *A. quadridentata* search for host eggs. They continuously tap the plant surface with their antennal tips until they contact a host egg (Rosenberg, 1934). The contact kairomone that mediates host-egg finding behavior is associated with host eggs and is water soluble, but its chemical structure has not yet been identified (Kromer, 1986).

Peculiar oviposition behavior by female *C. pomonella*, including brushing the surface of the leaf or fruit with the tip of her abdomen during oviposition (Borden, 1931), suggests that female moths may deposit body and wing scales on plants or eggs during the process of oviposition. Scanning electron micrographs revealed many scales from female moths attached to the surface of eggs from *C. pomonella* (Figure 3). Scales and volatiles from scales may constitute a reliable cue for foraging female *A. quadridentata* as they adhere to the egg surface, even when subjected to a fine airstream. Scales have been reported as a kairomonal source for other egg parasitoids (e.g., Lewis et al., 1972; Chiri and Legner, 1986), including *A. reticulatus* (Kainoh et al., 1990); therefore, we tested the hypothesis that scales from female *C. pomonella* are a source of kairomonal attraction for female *A. quadridentata*. Antennal responses from female and male *A. quadridentata* to scale volatile extract (Figure 2), wing fanning in the presence of scale volatiles followed by antennal tapping by females, and attraction of parasitoids to both natural (experiments 7 and 16) and synthetic scale volatile blends (experiment 8) all support the hypothesis that scale volatiles provide an important kairomonal cue for foraging females of *A. quadridentata*.

Response of female parasitoids to scale volatiles is likely of adaptive significance, focusing searching behavior on areas with high probabilities of encounter-

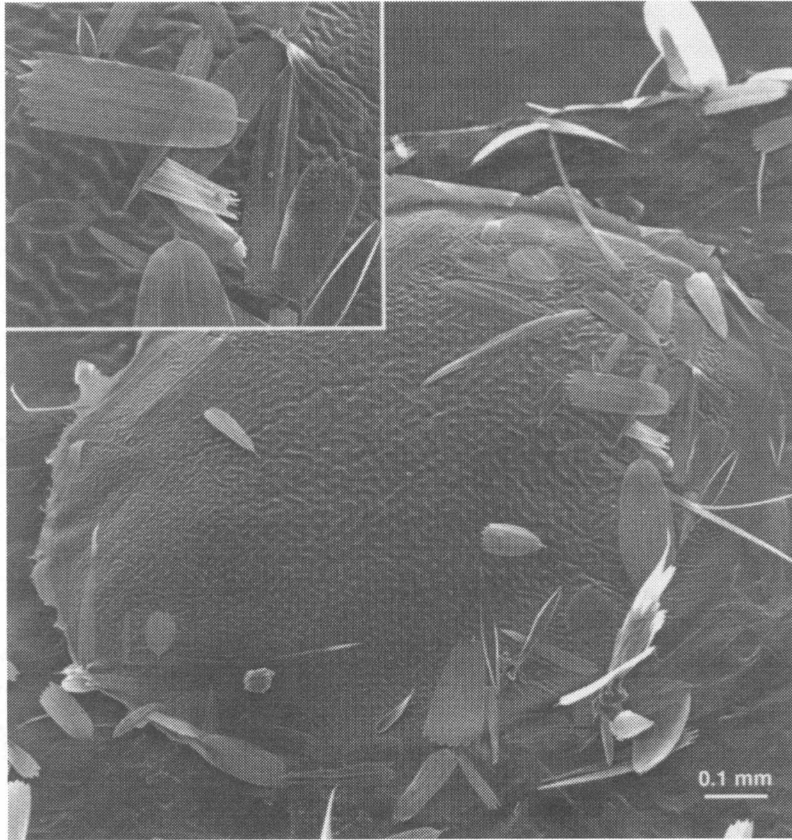

FIG. 3. Scanning electron micrograph of *C. pomonella* eggs, depicting moth scales embedded in the surface of the egg. Eggs were sputter-coated with gold, then viewed with a Hitachi S-2500 scanning electron microscope at 10 kV accelerating voltage.

ing host eggs (Chiri and Legner, 1982). By contrast, attraction of male parasitoids to scale volatiles (experiment 16) may have evolved to enhance the probability of mating with ovipositing female parasitoids.

Several volatiles in the synthetic kairomone blend attractive to female *A. quadridentata* (experiment 8) also have been reported as (potential) semiochemicals for other parasitoids. (*Z*)-9-Hexadecenal, for example, is a sex pheromone component of bollworm, *Heliothis zea* (Boddie), mediating kairomonal attraction of its egg parasitoid *Trichogramma pretiosum* Riley (Lewis et al., 1982). Heptanal elicits antennal responses from both male and female braconid *Microplitis croceipes* (Cresson) (Li et al., 1992). Habitat-derived nonanal is a synergis-

tic kairomone for *Apanteles carpatus* (Say), a parasitoid of clothes moth larvae (Takács et al., 1997). Nonanal in leaf volatiles from chestnut, *Castanea sativa* Miller, also elicits antennal responses from female *A. quadridentata*, although behavioral activity has not been established (Rotundo and Tremblay, 1993). Finally, octanal, nonanal, and decanal in abdominal gland secretions from stink bugs (Pentatomidae) may serve as kairomones for parasitic Tachinidae (Diptera) and Scelionidae (Hymenoptera) (Aldrich et al., 1995).

Sometimes combinations of semiochemicals from various sources are necessary to attract parasitoids (Nordlund et al., 1977; Godfray, 1994). For example, kairomonal attractiveness of *Trichogramma maidis* Pint. et Voeg., an egg parasitoid of the corn borer, *Ostrinia nubilalis* (Hübner), is based upon a complex mixture of odors comprising the corn borer's sex pheromone and volatiles from both the host egg and host plant (Kaiser et al., 1989). While scale volatiles of *C. pomonella* act alone as kairomonal attractants for male and female *A. quadridentata*, it should be investigated whether the sex pheromone of *C. pomonella*, egg volatiles, and/or apple volatiles, enhance the attractiveness of scale-derived kairomones.

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MANDIBULAR GLAND SECRETION IN DIFFERENT CASTES OF THE LEAF-CUTTER ANT *Atta laevigata*

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Abstract—Gas chromatography analyses and behavioral assays showed that *Atta laevigata*, a highly polymorphic ant species, has a mandibular gland secretion that varies with castes. All castes contain 4-methyl-3-heptanone as the main component and its concentration is proportional to head size. Small workers and soldiers, but not medium size workers, also contain 4-methyl-3-heptanol. Queens show variations in their chemical composition after mating, as virgin males contain a secretion dominated by 4-methyl-3-heptanol, and, in a lesser proportion, 4-methyl-3-heptanone. In mated males these proportions are inverted. The compounds 4-methyl-6-hepten-3-one, 4-methyl-4-hepten-3-one, 6-methyl-tetradecene, and 2,6-dimethyl-2-dodecene are found only in queens. The behavioral response elicited by the secretion is mainly alarm, which is elicited more strongly by glands of larger workers. The results suggest that chemical castes, behavioral castes, and morphological castes overlap in this species.

Key Words—4-Methyl-3-heptanone, 4-methyl-3-hexanone, 4-methyl-3-heptanol, castes, *Atta laevigata*, Formicidae, Attini, leaf-cutting ant, mandibular gland, behavior.

INTRODUCTION

The mandibular glands in the Formicidae are a source of volatile organic compounds. These compounds have strong effects on ant behavior (Cammaerts et al., 1983; Hölldobler and Wilson, 1990). Alcohols and their respective ketones are the chemical compounds most frequently found in the mandibular glands of

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Myrmicinae ants; many of these secretions have been analyzed and the results have been discussed and reviewed. The main function of the secretion from the mandibular glands is in signaling alarm behavior (Blum and Hermann, 1978; Parry and Morgan, 1979; Attygalle and Morgan, 1984; Hölldobler and Wilson, 1990).

Leaf-cutting ants are a serious problem, because they are widely distributed throughout the neotropics and are considered important pests in agricultural and arboricultural systems (Mayhé-Nunes, 1995; Cherrett and Peregrine, 1976; Hernández and Jaffe, 1995). These ants have a sophisticated communication system that is their main tool for the maintenance of the structure of their complex societies. Their colonies are strongly territorial (Jaffe et al., 1979; Vilela, 1983; Salzemann and Jaffe, 1990a,b) and the nestmate recognition system is based on odors present mainly in the head (Whitehouse and Jaffe, 1995), possibly from volatiles produced by the mandibular glands (Jaffe et al., 1979; Salzemann and Jaffe, 1991; Hernández and Jaffe, unpublished data). *Atta* colonies show a marked polyethism and polymorphism between members. Wilson (1980) divided *Atta sexdens rubropilosa* into four castes according to the tasks performed: gardener–nurses, generalists, forager–excavator, and defenders. These categories have raised questions regarding the chemical composition of the mandibular gland secretion in the different castes and the particular responses of the castes to this secretion (Do Nascimento et al., 1993).

The chemical composition of the mandibular gland secretion of other *Atta* species has been reported by several authors (Butenandt, 1959; Moser et al., 1968; Blum et al., 1968; Riley et al., 1974; Schildknecht, 1976), and almost all agree that the ketone 4-methyl-3-heptanone is the alarm pheromone in *Atta* species. Nonetheless, all these studies have been undertaken with massive extracts of all worker castes, without taking into account the strong polyethism and polymorphism present in the genus. Recently, Do Nascimento et al. (1993) reported the existence of variation between castes in the composition of the mandibular gland secretion of *Atta sexdens rubropilosa* and established that in the smaller worker caste the mandibular gland secretion is dominated by 4-methyl-3-heptanone, while in the larger worker caste this secretion is dominated by a mixture of neral and geranial. Virgin and mated queens contain mainly 4-methyl-3-heptanone, which increases in quantity after mating. Virgin males contain 4-methyl-3-heptanone and 4-methyl-3-heptanol in equal proportions, but in mated males the alcohol is absent.

Using the technique of individual gland analysis combined with gas chromatography–mass spectrometry (see Do Nascimento et al., 1993), we undertook chemical analysis of the mandibular gland secretion of the different castes of the ant *A. laevigata*. Additional experiments were performed in order to determine the possible behavioral relevance of the differences in chemical composition between castes.

METHODS AND MATERIALS

Ant Collection

Workers. Workers with different head widths were collected individually with a forceps from a colony located on the campus of the Universidad Simón Bolívar and were immediately introduced into 2- × 7-cm vials and placed in a container with Dry Ice. They were transported to the laboratory and stored at −20°C until analysis. Prior to the analysis the head width of each worker was measured.

Virgin Queens and Males. During the nuptial flights in June 1994, virgin females and males were collected as they left the ant nest at the pine tree plantations of CVG-PROFORCA, El Merey, Estado Anzoátegui, Venezuela. Each individual was treated in the same way as previously described for the collection of the workers.

Mated Females. Incipient colonies located in the pine tree plantations of CVG-PROFORCA were excavated and brought to the laboratory. Nests were kept at: 80–90% relative humidity, 24°C, 12L : 12D. The queens were kept with their respective workers until the analyses. Prior to the analyses, queen activity was slowed by cooling the insect to −20°C for 5 min.

Mated Males. Males were collected at the pine plantations as they landed from the nuptial flights in June 1995. The males were transported and stored until analysis in the same way as described for the workers.

Extracts

For all castes, each cephalic capsule was placed in a clean porcelain dish with 100 μl of hexane (pesticide grade, Fisher Scientific) and was crushed with a clean glass rod. One microliter was taken for analysis (see Do Nascimento et al., 1993). This method was carried out, because preliminary experiments showed that there were no differences between results from dissected mandibular glands extracts.

GC and GC-MS Analyses

Volatiles were analyzed by gas chromatography (GC). GC was performed on a Hewlett Packard 5890 Series II chromatograph attached to an HP 3396A integrator. The GC was equipped with a FID, a splitless injector, and a fused silica DB-5 capillary column (Quadrex, 25 m × 0.18 mm ID). The carrier gas was helium (flow rate 1 ml/min) and the oven was programmed with two temperature ramps, the first starting at 50°C, maintained for 4 min, and then increased to 150°C at 6°C/min. The second ramp started at 150°C, increased at 20°C/min to 280°C, and was then maintained at that temperature for 20 min. Compounds were identified by means of their mass spectra, with a Perkin-Elmer GC (Autosystem) coupled to a Perkin-Elmer MS (QMass-910). GC condi-

tions and column were the same as those used above. Identification of the compounds was achieved either by comparing their mass spectra with those from the NIST library and/or with synthetic standards synthesized in the laboratory or purchased commercially, or by comparison of the GC retention times of the standards. The purchased compounds were 4-methyl-3-hexanone, 3-heptanone, 2-heptanone, and 4-methyl-3-heptanol. 4-Methyl-3-heptanone was prepared from its alcohol by oxidation with pyridinium chlorochromate (PCC). Quantification was performed with 4-methyl-3-penten-2-one as an external standard. This compound was chosen as standard because it has a relative FID response factor similar to all the other identified compounds. For each caste, five replicates were undertaken, and all data were analyzed by means of one-way ANOVA (Siegel and Castellan, 1988) with SPSSPC.

Behavioral Bioassays

Behavioral tests were performed on colonies kept in the laboratory. Tests were undertaken on a foraging area, which consisted of a plastic container (18 cm high, 60 and 40 cm upper and lower diameter). In the bottom of the container we placed fine cardboard (white) with concentric circles drawn on it in order to aid in estimating the distance between the ant showing a particular behavior and the odor source. Over the cardboard a glass sheet (40 cm diam.) was placed to facilitate cleaning the substrate of the arena for each bioassay. As foraging substrate, leaves of *Hura crepitans* were placed daily on the foraging area so that ants could recognize the foraging arena as a colony territory (Salzemann and Jaffe, 1991). We then presented different odors to workers foraging on this arena. The odors were body parts taken from live queens and workers of *A. laevigata*, crushed on a glass sheet (40 cm diam.) with a clean glass rod. Each test was filmed for 10 min with a video camera (SONY Handycam CCD-F360). Films were used to quantify the behavior of workers elicited during the first 5, 15, 30, 45, and 60 sec after presenting the odor and then during 15 sec for each minute up to minute 10. In each observation we determined the percentage of ants exhibiting a specific behavior. Controls were lyophilized worker and queen bodies, submitted to high vacuum at $-20°C$ for 26 hr (Freeze Dryer 4.5, Labconco). Castes were classified according to their head width.

RESULTS

Chemical Analyses

Cephalic capsules of 35 ants belonging to different castes of *A. laevigata* were analyzed. The compounds identified and the average compositions nanograms per head for each caste are given in Table 1. The different castes showed differences in the chemical composition and the relative proportions

TABLE 1. VARIATION OF COMPOSITION AND ABSOLUTE QUANTITY OF MANDIBULAR GLAND SECRETION IN DIFFERENT CASTES OF *A. laevigata*[a]

Compounds	Virgin queen	Mated queen	Virgin male	Mated male	Soldier	Worker 2.5–4 mm	Worker <2.5 mm	χ^2	*P*
1) 4-methyl-3-hexanone	14 ± 13	3.5 ± 5		1.5 ± 1.4	1.7 ± 1.5	0.3 ± 0.4	0.02 ± 0.1	15.8	0.002
2) 3-methyl-2-hexanone	t	t							
3) 4-methyl-3-hexanol	t	t							
4) 3-heptanone	t	t					t		
5) 2-heptanone	t						t		
6) 4-methyl-6-hepten-3-one	0.6 ± 0.6	0.1 ± 0.2					t	15.2	0.018
7) 4-methyl-3-heptanone	1013 ± 162	167 ± 120	3.4 ± 2.8	51 ± 47	16 ± 12	4.5 ± 2.6	1.9 ± 0.5	27	<0.001
8) 4-methyl-3-heptanol	9 ± 3	1.8 ± 1.3	48 ± 36	2.7 ± 1.5	0.4 ± 0.4	t	1.1 ± 1.5	23.8	<0.001
9) 3-octanone	0.4 ± 0.4	1.1 ± 2.6				t		29.4	<0.001
10) 4-methyl-4-hepten-3-one	1.2 ± 0.2	0.2 ± 0.5						4.4	0.642
11) 6-methyl-tetradecene	3.0 ± 3.1	1.3 ± 2.3						15.4	0.017
12) 2,6-dimethyl-2-dodecene	26 ± 8							35	<0.001

[a]Kruskal-Wallis ANOVA if $P < 0.05$. The values are the mean of the relative abundance of compounds (ng/head ± SD). $N = 5$ for all replicates; t = trace compounds.

of compounds present in their mandibular gland secretion. In all castes, except virgin males, 4-methyl-3-heptanone was the main component of the secretion. Queens had more compounds than other castes, with their secretion dominated by the 4-methyl-3-heptanone, 4-methyl-3-hexanone, and 4-methyl-3-heptanol. In addition queen mandibular glands contained 3-methyl-2-hexanone, 4-methyl-3-hexanol, 3-octanone, 4-methyl-4-hepten-3-one, 3-methyl undecene, and 6-methyl tridecene, which were absent in all other castes, or if present in other castes, in quantities we were unable to detect. The relative abundance of the compounds in mandibular glands from virgin queens differed markedly from that of mated queens (Table 1). Mandibular glands of virgin males contained only 4-methyl-3-heptanone and 4-methyl-3-heptanol, the latter compound being the most abundant in the secretion. After mating, the relative proportion of both compounds was inverted, and in addition 4-methyl-3-hexanone was detected. We observed a decreasing concentration of all the compounds identified linked to decreasing head width in the castes. In virgin males (head width >2.36 mm), however, the amount of 4-methyl-3-heptanone contained in the secretion was greater than that of both workers and soldiers. Mandibular glands of soldiers contained 4-methyl-3-hexanone, 3-heptanone, 4-methyl-3-heptanone, and 4-methyl-3-heptanol. Workers, independent of head width, also produced 4-methyl-3-hexanone and 4-methyl-3-heptanone (Table 1). 4-Methyl-3-heptanol was not, however, observed and the 3-octanone was only detected in trace amounts. We also identified with GC-MS analysis the compounds 3-hexanone, 2-hexanone, 3-hexanol, 2-hexanol, hexane-3-hydroxyperoxy, and hexane-2-hydroxyperoxy in the extracts of the mandibular glands of all castes; however, these compounds are artifacts produced by the reaction of the samples with the hexane (W. Francke, personal communication). The results presented here are statistically significant, as an ANOVA of the analyzed samples showed statistically significant differences in the composition and relative abundance of the volatile compounds found in the mandibular glands between the castes analyzed (Table 1).

Behavioral Bioassays

Tests presenting the different body parts to ants colonies allowed us to characterize the following behaviors:

Attraction. Ants orient toward the source (body part presented) and walk (move more than half their body length per second) or run (move more than half their body length per sec) toward it.

Alarm. Ants detecting the source open their mandibles, bend their gaster ventrally, and run in irregular circles around the source.

Antennation. Ants touch the source with the tip of their antennae, pausing for more than 1 sec at the source.

Attack. Ants stand in front of the source with their bodies tilted upwards ante-

riorly and with their mandibles open. Then they quickly rock forward towards the source pivoting on their back pair of legs and quickly return to their original position. Occasionally they close their mandibles at the most anterior point of the cycle, opening them again as they rock back to their original position.

Biting. Ants grasp the source with their mandibles and pull backwards, some times dragging the source.

Trembling. Ants vibrate rapidly (i.e., shiver) and sometimes press their bodies against the ground.

Grooming. Ants groom appendages by either passing them through their mouthparts or by repeatedly rubbing a leg against it.

Alert. Ants stop and lift their head, thorax, and antennae.

Pause. Ants stand with their legs more or less straight and stiff so that their bodies are held high above the substrate. The thorax of each ant is slightly tilted upward anteriorly while the head is strongly tilted upwards. The gaping mandibles are almost perpendicular to the substrate.

Transport. Ants pick up the source with their mandibles and move it off the trail (in the field) or carry it to the waste (in the laboratory).

In tests presenting the different body parts to ants in colonies, only a few of the behaviors detected were analyzed because of the low frequency of occurrence of most of them. Tests revealed that during the first minute of presentation, the head of the queen was the body segment that elicited the most behaviors and the strongest response in workers, followed by the head of workers. Among those behaviors, only alarm behavior showed statistically significant differences among castes (Figure 1A and B). The differences in alarm elicited by the head of queens compared with that elicited by the thorax and abdomen was highly significant (chi-square test, $P < 0.0001$). Among body segments of workers, head odors elicited the strongest alarm response in workers followed by the gaster. The thorax did not elicit significant behavioral responses (Figure 1B). Behavior decreased during the 10-min observation period in all worker castes (Figure 2). During the first 2 min of evaluation, alarm was the preponderant behavior but attack behavior was also observed in frequencies that varied between 2 and 8% of workers.

DISCUSSION

Wilson (1980) reported the existence of four morphological and behavioral castes in workers of the ant *Atta sexdens rubropilosa*. Our observations suggest a similar polymorphism in *A. laevigata*. Chemical composition of the mandibular glands of the castes differ. However, our results are very different from those reported for *A. sexdens rubropilosa* (Do Nascimento et al., 1993). In that ant, only the smallest workers (head width < 1 mm) contain the compound 4-methyl-3-heptanone as the main component of the mandibular gland secretion,

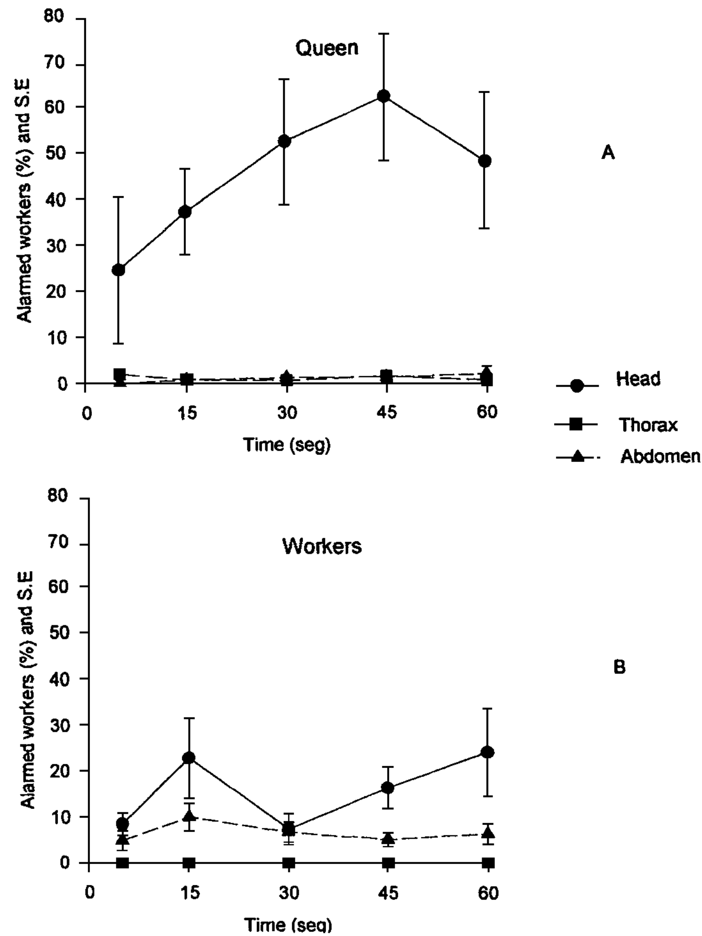

FIG. 1. (A) Alarm behavior produced in forager workers of *Atta laevigata* by odors from crushed queen's body parts. (B) Alarm behavior produced in forager workers of *Atta laevigata* by odors from crushed worker's body parts.

while the dominant compound in larger workers was citral. In *A. laevigata*, all castes, except virgin males, contain 4-methyl-3-heptanone as the main component. The fact that in workers the relative proportions of 4-methyl-3-hexanone and 4-methyl-3-heptanone decreased with head width is congruent with morphological studies of the mandibular glands of *Atta laevigata* (Hernández and Caetano, 1995). These authors reported that the size of the glands is proportional to the size of the cephalic capsule. It is known that the same compound

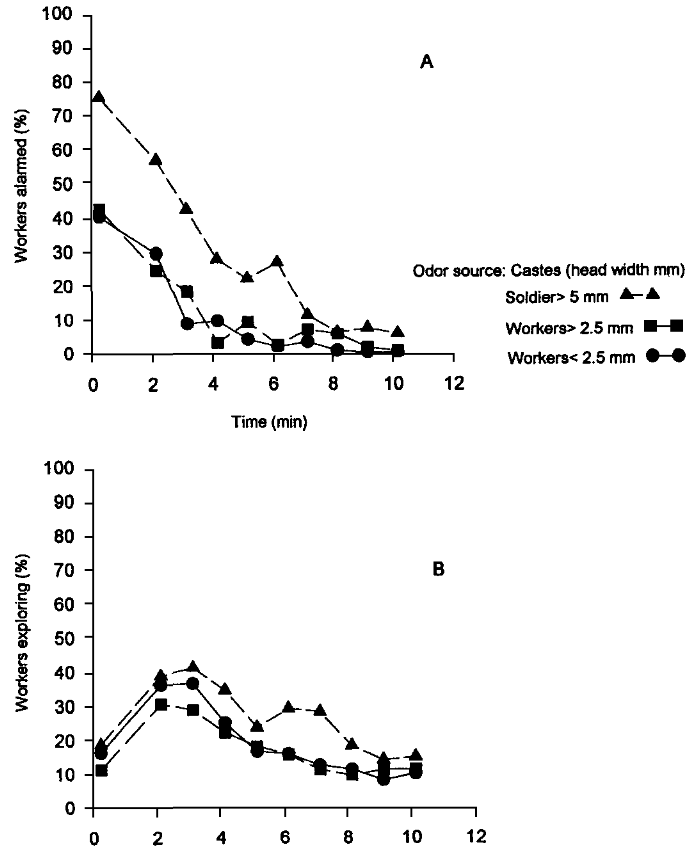

FIG. 2. (A) Alarm behavior produced in forager workers of *Atta laevigata* by odors from crushed head from workers of different castes. (B) Exploring behavior produced in forager workers of *Atta laevigata* by odors from crushed head from workers of different castes.

can release different behaviors in ants depending on its concentration (Moser et al., 1968; Blum, 1996). In leaf-cutting ants, each worker caste is involved in different activities within the colony (Wilson, 1980). A single compound may also release different behaviors depending on the context of the signal. For example 4-methyl-3-heptanone could be used by workers as an alarm signal, and the same compound may be used by females and males as mating cues.

The chemical composition of the mandibular gland secretion in males and queens of *A. laevigata* is different from results reported for *A. sexdens rubropilosa* (Do Nascimento et al., 1993). The facts that in *A. laevigata* the amount of

secretion in males and queens decreases after the nuptial flight and that males show a very different composition compared to queens suggest that these compounds may be used in the communication between the sexes. Do Nascimento et al. (1993) suggested that *A. sexdens rubropilosa* uses the alcohol as an attractant for mating, which could also be the case in *A. laevigata*. Nonetheless, these authors do not explain the high quantities of 4-methyl-3-heptanone found in mated males. The reason for high amounts of 4-methyl-3-heptanone in males after the nuptial flight is not clear, given the short time they survive after mating. It is possible that 4-methyl-3-heptanol can be converted to the ketone by means of oxidation. Thus, males may synthesize the ketone from the alcohol as a precursor during the nuptial flight, and use this ketone for communication, resulting in a change in the mandibular gland chemistry between virgin and mated males.

Wilson (1980) proposed that the smaller worker castes in *Atta sexdens rubropilosa* are more primitive than higher castes. He based this hypothesis on the fact that they are morphologically more similar to the less modified monomorphic members in the same genus and that they carry out fewer activities within the ant society, acting only as nurses and gardeners within the nest. Do Nascimento et al. (1993) supported this hypothesis based on the fact that workers in the smaller castes contain far fewer chemical compounds in the mandibular gland than workers in larger castes. We found that the chemical composition of the mandibular gland is more or less similar in all worker castes in *Atta laevigata*, except for the medium size caste, which according to the primitive caste hypothesis would be primitive caste. Our results suggest the existence of chemical castes that seem to overlap with the caste system proposed by Wilson (1980) based on morphological and behavioral characteristics.

Jaffe (1982, 1987) suggested that nestmate recognition is achieved by the odors produced by the mandibular glands, with respect to the nestmate recognition system in *Atta* ants. Our results suggest the possibility that the mandibular gland secretions could be used by workers to differentiate the castes. These compounds are released continuously in low quantities and are adsorbed by cuticular hydrocarbons over the ant's body.

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EXPERIENCE-DEPENDENT MODIFICATION OF ORIENTATIONAL RESPONSE TO OLFACTORY CUES IN LARVAE OF *Spodoptera littoralis*

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Abstract—In a behavioral dual-choice test the orientational response of third-instar larvae of *Spodoptera littoralis* to a certain odor could be increased, following a former experience of the odor. The odorants used were either the odor of a previously eaten host plant or a synthetic plant odor presented in conjunction with food. Inexperienced third-instar larvae were either neutral or demonstrated a weak attraction to the odors, whereas experienced larvae were highly attracted. Furthermore it was demonstrated that the larvae did not generalize between the odor previously experienced and a novel odor.

Key Words—*Spodoptera littoralis*, larvae, olfaction, orientation, induction.

INTRODUCTION

A previous food experience by lepidopterous larvae can influence food preference at a later stage. This phenomenon has been called induction of feeding preference by Jermy et al. (1968). The mechanisms of induction are unknown and do not fit into the usual categories of learning (Dethier, 1982). Induced feeding preferences are common in insect larvae and not restricted to certain taxonomic groups (for review see Jermy, 1987). However, it is not completely universal. Chew (1980) reported lack of induction in *Pieris rapae* and *P. napi*.

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Preference for a certain host plant could be induced in larvae of *Manduca sexta* after only one day of feeding experience, and the preference was not lost after several moltings (Jermy et al., 1968). *M. sexta* and other oligophagous species have demonstrated innate preference hierarchies among hosts and nonhosts (Barbosa et al., 1979; de Boer and Hanson, 1984). These species may, after rearing on a nonhost plant, increase the preference for this plant but the preference for the true host plant does not diminish (Barbosa et al., 1979; de Boer and Hanson, 1984). In the early induction experiments larvae were allowed close contact with the plants when discriminating between them, and the degree of induction was measured as consumption. Whether an induced preference was due to an increased feeding intensity when contacting the food or an increased distance attraction is not clear. However, it was shown in ablation experiments that both olfaction and gustation mediate important information in food plant discrimination by lepidopterous larvae (Hanson and Dethier, 1973; de Boer and Hanson, 1987).

An increase in orientational response to certain food types was first demonstrated in *Manduca sexta*, an oligophagous species, by Saxena and Schoonhoven (1978, 1982). In their experiments, the larvae showed orientational preference for the diet on which they had been cultured. They also demonstrated an orientational preference for a diet containing an incorporated synthetic odorant that they had experienced earlier compared to the same diet without odorant. However, the behavioral preference could be for the combination of diet and odorant rather than for the odorant itself.

The aim of our study was to examine the modification of orientational response in larvae of a polyphagous species, the Egyptian cotton leafworm (*Spodoptera littoralis*, Boisd.), to odors that had previously been experienced. The odorants were either the odor of a previously eaten host plant or a synthetic plant odor presented in conjunction with feeding but not incorporated in the food.

S. littoralis is a highly polyphagous species. It has been found to feed on at least 84 plant species within 40 families (Brown and Dewhurst, 1975). In this species, larval feeding has been shown to be influenced by previous feeding experience (Anderson et al., 1995). Furthermore, adult moths of *S. littoralis* have demonstrated an ability to learn and memorize floral odors associated with a food reward in classical conditioning experiments (Hartlieb et al., unpublished data; Fan et al., 1997).

METHODS AND MATERIALS

Insects. Larvae of *S. littoralis* were taken from a culture reared for several generations on a synthetic diet (Hinks and Byers, 1976). The culture originates from a laboratory culture at SLU in Alnarp, Sweden. Wild collected moths have

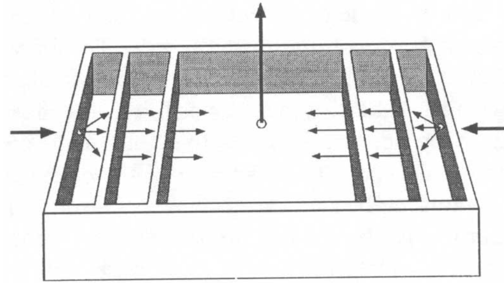

FIG. 1. A schematic drawing of the larval olfactometer. The box contains five internal chambers. The four distal chambers served as air baffles. Air currents were drawn bidirectionally through the central chamber by applying a vacuum in the center of the cover of the box as shown in the figure. To test orientational responses to test odors, the larvae were dropped on a midline of the central chamber. The converging air currents provided the larvae with a choice between fresh air and odor.

been introduced into the culture at several occasions during the last five years. Egg batches were transferred to boxes containing either a synthetic white bean diet (Zhu et al., 1996) or leaves of cotton, *Gossypium hirsutum*. The larvae were kept at 23°C, 70% relative humidity, and a 16L : 8D cycle and supplied with an excess of food until used in the experiments.

Bioassay. Orientational response of larvae to test odors was assayed in a dual-choice olfactometer (Figure 1), where the larvae had a choice between fresh air and the test odor. The olfactometer was a modified version of a test chamber used for conditioning fruitflies (Tully et al., 1994). A central chamber (200 × 200 mm) served as the experimental arena, whereas two chambers on each side of the central arena served as air baffles. Porous metal walls separated the distal chambers from each other and nylon meshes separated the central chamber from the air baffles. Convergent air currents were drawn through the box by applying a vacuum in the center of the cover. The vacuum was adjusted to let air flow at 1000 ml/min from both sides. A smokescreen test revealed that this speed created laminar flow in two distinct air parcels in the chamber. Air was charcoal-purified, humidified in distilled water, and finally drawn through either a control bottle or a bottle containing an odorant.

Groups of 10–20 larvae were placed on the midline of the central chamber and were exposed to converging air currents. After 5 min (10 min for newly hatched larvae) the number of larvae on either side of the midline were counted. Larvae that stayed motionless during the entire test period were not counted. A performance index (PI) was calculated according to the formula:

$$PI = [(SS - NSS)/(SS + NSS)] \times 100$$

where *SS* is the number of larvae at the end of the experiment on the odor side and *NSS* the number of larvae on the fresh air side. The *PI* would be zero if equal numbers of larvae were found on each side of the chamber and ±100 if all larvae preferred one side of the chamber. A positive value indicated a majority of the larvae on the odor side, while a negative value indicated the converse. Each larva was tested only once. The odor side of the chamber was randomly altered to exclude phototactic behavior as a source of error. Between replicates the chamber was thoroughly cleaned to eliminate residual scent. Before testing, all larvae were starved overnight (16–20 hr) to increase their motivation.

Experiment 1: Attraction to Cotton Leaves. Attraction to cotton leaf odor was tested in newly hatched larvae and in third-instar larvae reared on cotton leaves. A large intact leaf of cotton (3 ± 0.2 g) was placed in a bottle, over which a current of air was drawn. Larvae with no prior experience of cotton leaves (reared on synthetic diet) served as a control. To test the persistence of an induced attraction to cotton leaf odor, the diet was switched for a group of third-instar larvae from cotton leaf diet to a synthetic diet at 24 hr prior to testing. A fourth group of larvae reared on synthetic diet was transferred to and allowed to feed on cotton leaves 24 hr prior to the starvation period prior to testing to determine whether an attraction could be induced in a shorter period of time.

Experiment 2: Attraction to Geraniol. In a second series of experiments the larvae were exposed to the odor of geraniol (Chemicon, Sweden) while feeding on a synthetic diet. A dose of 1000 μg (0.5 μg/ml acetone solution) was applied to filter papers (90 mm ID). After evaporation of the solvent, the filter paper was placed in the larval rearing box in order to scent the atmosphere of the box with geraniol. The filter papers used in the olfactometer were impregnated with a 10 times higher concentration than in the rearing boxes.

The lid of a Petri dish, with a hole (70 mm ID) cut in the center covered with nylon mesh, served as the feeding site in the rearing chambers. Odor-impregnated filter papers were placed underneath the mesh of the petri dish and the synthetic diet was placed on top of it. The control group was reared in the same way but the filter papers were impregnated with solvent only. Filter papers were changed daily.

The behavioral response to the odor of geraniol was tested on newly hatched larvae and on third-instar larvae with or without experience of the odor. Retention of a modified orientational response to geraniol was tested by transferring previously exposed larvae to a nonscented environment for 24 hr. To determine whether behavior could be modified in a shorter period of time, third-instar larvae with no prior experience of geraniol were exposed to the odor in conjunction with feeding for 24 hr before testing.

Experiment 3: Generalization. To test whether larvae generalized between the odor previously experienced and a novel odor, third-instar geraniol-experienced larvae were tested on another plant odor, linalool (Chemicon, Sweden).

The orientational response of these larvae was compared with larvae exposed to linalool during feeding and larvae with no experience of either linalool or geraniol.

Data Analysis. Within each group a chi-squared test was performed to test if the response to the odor differed from zero. For differences between groups, a one-way analysis of variance (ANOVA) test with a subsequent Fisher's PLSD post hoc test was used, comparing means of performance indices.

RESULTS

Attraction to Cotton Leaves. Newly hatched larvae and third-instar larvae with prior experience only of synthetic diet were not attracted to the odor of intact cotton leaves ($P > 0.05$, χ^2). Third-instar larvae fed on cotton leaves since hatching demonstrated a significant increase in orientational response compared to newly hatched larvae and inexperienced third-instar larvae (Figure 2, columns a–c; $P < 0.05$, ANOVA followed by Fisher's PLSD test). The response of third-instar larvae, previously reared on synthetic diet but given the opportunity to feed on cotton leaves for 24 hr, differed significantly from the response of inexperienced third instars (Figure 2, columns b and d; $P < 0.05$, ANOVA followed

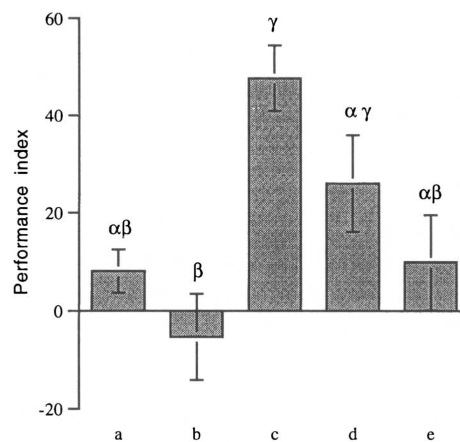

FIG. 2. Response to the odor of intact cotton leaves expressed as mean PI ± SEM in (a) newly hatched larvae, (b) synthetic diet-reared third instars, (c) third instars with lifelong experience of cotton leaves, (d) third instars with only 24 hr of experience of cotton leaves prior to testing, and (e) third instars with lifelong experience of cotton leaves but fed on synthetic diet 24 hr prior to testing. Bars capped by different letters differ significantly ($P < 0.05$, ANOVA followed by Fisher's PLSD test). Each group is represented by 10 replicates with 10–15 larvae.

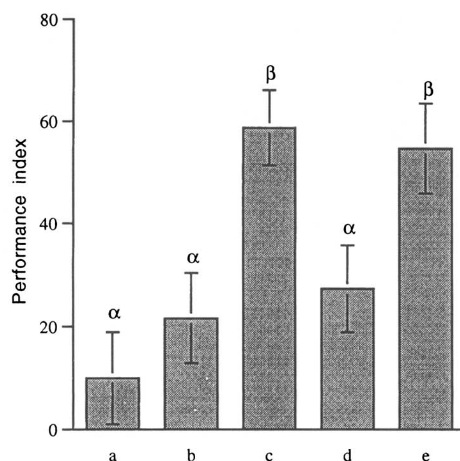

FIG. 3. Response to the odor of geraniol expressed as mean PI ± SEM in (a) newly hatched larvae, (b) inexperienced third instars, (c) experienced third instars, (d) third instars with only 24 hr of experience of geraniol prior to testing, and (e) experienced third instars with no odor exposure 24 hr prior to the starvation period. Bars capped by different letters differ significantly ($P < 0.05$, ANOVA followed by Fisher's PLSD test). Each group is represented by 10 replicates with 10–15 larvae in each.

by Fisher's PLSD test). Another group of larvae was transferred in the opposite direction and given synthetic diet for 24 hr after previously being fed with cotton leaves. The response of these larvae did not differ from the response of inexperienced larvae (Figure 2, columns b and e; $P > 0.05$, ANOVA followed by Fisher's PLSD test).

Attraction to Geraniol. In a second series of experiments the larvae were exposed to geraniol in conjunction with feeding on a synthetic diet. Newly hatched larvae did not respond to the odor of geraniol ($P > 0.05$, χ^2), whereas all other groups showed a preference for geraniol over fresh air ($P < 0.05$, χ^2). Larvae exposed to geraniol in conjunction with feeding showed a significantly higher attraction to the odor than inexperienced larvae (Figure 3, columns b and c; $P < 0.01$, ANOVA followed by Fisher's PLSD test). Exposure for 24 hr to geraniol in third instars previously reared without exposure did not elicit any stronger response than in inexperienced larvae (Figure 3, columns b and d; $P > 0.05$, ANOVA followed by Fisher's PLSD test). The increased orientational response acquired during the first three instars did not decrease after 24 hr of feeding without exposure to geraniol (Figure 3, columns c and e; $P > 0.05$, ANOVA followed by Fisher's PLSD test).

Generalization Test. The generalization test demonstrated that third instars exposed to geraniol during feeding and larvae not exposed to any synthetic odors

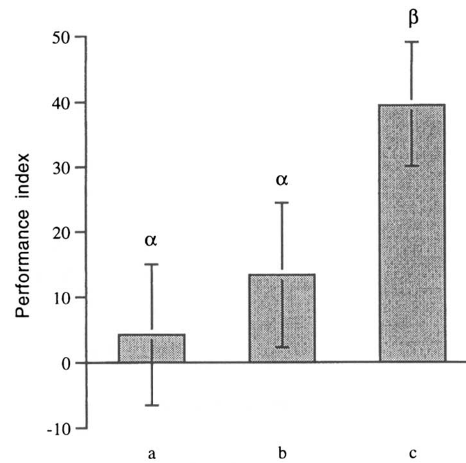

FIG. 4. Response to the odor of linalool expressed as mean PI ± SEM in (a) third instars reared without exposure to geraniol or linalool, (b) third instars exposed to geraniol, and (c) third instars exposed to linalool. Bars capped by different letters differ significantly ($P < 0.05$, ANOVA followed by Fisher's PLSD test). Each group is represented by 10 replicates with 10 larvae in each.

while feeding did not show as strong attraction to linalool, another plant odor, as larvae previously exposed to that odor (Figure 4; $P < 0.05$, ANOVA followed by Fisher's PLSD test). Thus, the larvae did not generalize between the odor experienced and a novel flower odor.

DISCUSSION

The present study clearly demonstrates that an orientational response to a host plant can be induced in larvae by previous feeding experience of the plant. Similar results were found by Saxena and Schoonhoven (1978, 1982), who showed that induction of feeding preference not only leads to an increased feeding intensity, but also to an increase in orientational response. The construction of our olfactometer and the one used by Saxena and Schoonhoven excludes involvement of other sensory modalities than olfaction in orientation. Additionally, our experiments demonstrate that increase of orientational response to an odor is not restricted to the food source itself or an additive in an artificial diet. A synthetic plant compound presented in conjunction with feeding, but not added to the food, also elicited an increased orientational response in experienced larvae.

Larvae reared on a synthetic diet did not show any attraction to the odor of

cotton leaves, whereas cotton-reared larvae were highly attracted. Attraction in third instars could be induced after only 24 hr of feeding. This is in accordance with earlier induction experiments (Jermy et al., 1968) where a 24-hr feeding experience was sufficient to induce a host plant preference. However, attraction to cotton leaf odor was lost after the larvae were transferred from previously feeding on cotton leaves to a synthetic diet. In *M. sexta*, it has been shown that an induced feeding preference persists through several larval molts (Jermy et al., 1968). A major difference between that species and *S. littoralis* is the range of host-plant acceptability. Whereas the range of *M. sexta* is restricted to plant species within the family Solanaceae (Yamamoto and Fraenkel, 1960), *S. littoralis* is a highly polyphagous species accepting plants within at least 40 families (Brown and Dewhurst, 1975).

The absence of response to cotton leaf odor in inexperienced larvae is in contrast with the findings by Khalifa et al. (1973), who demonstrated that cotton leaves were highly attractive to third-instar larvae of *S. littoralis*. However, in the experiments by Khalifa et al. (1973), the larvae were confronted with extracts of ground leaves and not with intact leaves, and the diet on which the larvae had been reared is not mentioned in the report. Furthermore, the larvae were allowed close contact with the extracts, and therefore the involvement of sensory modalities other than olfaction cannot be excluded.

Newly hatched larvae of *S. littoralis* did not respond either to cotton leaf odor or to geraniol in our experiments, whereas newly hatched larvae of *M. sexta* were strongly attracted to the odor of host plants (Saxena and Schoonhoven, 1982). An innate preference hierarchy among host plants has been demonstrated in oligophagous species (Barbosa et al., 1979; de Boer and Hanson, 1984). As discussed below, a polyphagous species may not be equipped with an innate response to host plants.

Just as in the cotton diet experiment, a previous exposure to geraniol in conjunction with feeding resulted in an increased attraction to geraniol compared with inexperienced larvae. However, unlike the former experiment, 24 hr of exposure to geraniol in third instars was not sufficient to alter the response to geraniol. Neither did the response decrease in larvae previously exposed to geraniol but allowed to feed without exposure for 24 hr. The differences in the experiments may be due to the diet switch from synthetic diet to cotton leaves that could have induced a faster modification of behavior. In the second experiment only the odor associated with the synthetic diet was added or deducted.

A modified orientational response after a previous experience was restricted to the experienced odor, geraniol, and not a nonexperienced plant odor, linalool. This shows that the larvae can discriminate between the odorants and, furthermore, do not generalize between the two plant odors. In addition, this test demonstrated that modification of the behavioral response after experience was not restricted to the odor of geraniol. A previous experience of linalool in conjunc-

tion with feeding induced an increased response to linalool comparable to that observed with geraniol.

A reasonable explanation for the increased orientational responses to the previously experienced odors is that the larvae associate the odors with a potential food source. Habituation to deterring compounds in cotton leaves is another possible explanation for the acquired olfactory attraction to the leaves. Habituation to feeding deterrents (Jermy, 1987) and to oviposition deterrents (Jaenike, 1988; Anderson et al., 1995) has been demonstrated. In these experiments a previous exposure to the deterring compounds reduced the aversion towards them. This explanation can be excluded in our second experiment, as geraniol is a single compound that elicited a response even in inexperienced third-instar larvae. A relation to gut content can also be excluded in the second experiment as no direct contact with the odor source was established.

Whether the larvae associate geraniol or linalool with the food they have been cultured on is not clear. It cannot be ruled out that a modification of the orientational response would occur even if the odors were not presented in conjunction with feeding. It may be possible to test for this by exposing the larvae to odors at limited time periods with or without association with food. The exposure periods should be short enough to avoid effects of starvation for the nonassociated group.

It is very unlikely that a larva of a highly polyphagous species such as *S. littoralis* will encounter all potential host plants during larval development. An innate behavioral response to the odor of all these plants would therefore be an extravagance. The larvae would probably be better served by an ability to modify their behavior after having experienced a novel food source. This would enable the larvae to test a wide range of plants and either accept or reject them as suitable food. An ability to induce a preference for an odor associated with a food source would most likely simplify foraging.

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SHIFTS IN LIFE HISTORY AS A RESPONSE TO PREDATION IN WESTERN TOADS (*Bufo boreas*)

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Abstract—Larval western toads (*Bufo boreas*) are known to exhibit antipredator behavior in response to both chemical alarm cues released from injured conspecifics and chemical cues of predatory invertebrates. In this study, we tested whether long-term exposure to predator and alarm cues resulted in an adaptive shift in life history characteristics of the toads. We raised groups of tadpoles in the presence of: (1) predatory backswimmers (*Notonecta* spp.) that were fed toad tadpoles, (2) nonpredatory water boatman (Corixidae), and (3) chemical alarm cues of injured conspecifics. Tadpoles raised in the presence of both chemical alarm cues and cues of predators fed tadpoles metamorphosed in significantly shorter time than those raised in the presence of the nonpredator control. Reducing time taken to reach metamorphosis would reduce exposure to aquatic predators. There was no difference among treatments in the size at metamorphosis. Our results suggest that this shift in metamorphic characteristics may represent a facultative alteration in life history.

Key Words—Predation, life history, chemical cues, alarm cues, amphibians, western toads, *Bufo boreas*.

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INTRODUCTION

Predation is a strong selective force influencing the behavior, morphology, and life history of prey species (Sih, 1987; Lima and Dill, 1990; Chivers and Smith, 1998). Most studies of prey defenses have concentrated on behavioral or morphological defenses. Fewer studies have examined the effects of predation on alterations in life history characteristics (patterns of growth and reproduction).

Amphibians provide a model system for studying the effects of predation on life history shifts (see Werner, 1986). For example, Skelly (1992) found that gray treefrog (*Hyla versicolor*) tadpoles had reduced growth and development rates when exposed to caged larval tiger salamanders (*Ambystoma tigrinum*). Skelly and Werner (1990) demonstrated that larval American toads (*Bufo americanus*) metamorphosed at a smaller size in the presence of dragonfly (*Anax junius*) predators than in their absence. Van Buskirk (1988) and Wilbur and Fauth (1990) documented that American toad tadpoles metamorphosed earlier and at a smaller size in the presence of dragonfly predators.

Few studies have examined the importance of chemical cues as signals inducing changes in amphibian life history traits (Kats and Dill, 1998). In one study, Sih and Moore (1993) demonstrated that salamanders (*Ambystoma barbouri*) delayed hatching in the presence of chemical cues of predatory flatworms (*Phagocottus gracilis*), but not in response to cues of nonpredatory isopods (*Lirceus fontinalis*). In another study, larval long-toed salamanders (*Ambystoma macrodactylum*) exhibited slower growth and an increase in time to reach metamorphosis in the presence of conspecific predators fed a cannibal diet over conspecifics fed an invertebrate diet (Wildy et al., 1999).

Behavioral responses of larval amphibians to chemical cues are widespread (reviews Chivers and Smith, 1998; Kats and Dill, 1998). For example, several bufonid tadpoles, including those of the western toad, exhibit antipredator behavior to chemical alarm cues released from injured conspecifics (Pfeiffer, 1966; Hews and Blaustein, 1985; Hews, 1988; Petranka, 1989). The specific chemical that acts as the alarm cue for bufonid tadpoles may be bufotoxin (Kulzer, 1954). Bufonid tadpoles also respond to chemical cues of potential predators. For example, Kiesecker et al. (1996) demonstrated that western toad tadpoles exhibit antipredator behavior in response to chemical cues of predatory backswimmers (*Notonecta* spp.), giant waterbugs (*Lethocerus americanus*), and garter snakes (*Thamnophis sirtalis*). Chemical cues are of prime importance in recognition of insect predators by western toad tadpoles. Tadpoles respond to chemical but not visual cues of predatory backswimmers and giant waterbugs (Kiesecker et al., 1996).

In this study, we examined the effects of predation risk on life history characteristics of western toads (*Bufo boreas*). We raised tadpoles in the presence of predatory backswimmers, nonpredatory water boatman, or chemical cues from

injured conspecifics to test whether the tadpoles change characteristics of their life history in response to predation cues. Specifically, we tested whether the risk of predation alters the time it takes tadpoles to reach metamorphosis or the size the individuals attain upon reaching metamorphosis.

METHODS AND MATERIALS

We collected larval toad tadpoles from Lost Lake, Linn County, Oregon (44°26′42″N, 121°55′30″W) in the summer of 1996. The tadpoles were transported to Oregon State University for testing. Prior to beginning experiments, tadpoles were maintained in 37-liter glass aquaria on a 14L : 10D photoperiod at approximately 20°C. The tadpoles were fed ad libitum with ground alfalfa pellets.

We divided 12 glass aquaria widthwise with a fiberglass mesh screen to create two compartments, each measuring 25 × 30 × 25 cm. Twelve randomly selected tadpoles were placed onto one side of each of the 12 test tanks. All tadpoles were at the same stage of development (Gosner stage 25) (Gosner, 1960) at the beginning of the experiment. An additional three tadpoles were placed onto the opposite side of each tank from where the test tadpoles were placed. Our experiment consisted of raising groups of tadpoles under three different treatments in a randomized block design with four replicates of each treatment. Treatment 1 was a predator treatment. In this treatment we placed three predatory backswimmers into each aquarium. The predators were placed at the stimulus end of the tank (i.e., the end opposite from where the 12 test tadpoles were housed). Treatment 2 was a nonpredator treatment in which we placed three nonpredatory water boatman into the stimulus end of each aquarium. Treatment 3 consisted of exposing test tadpoles to alarm cues from injured conspecifics.

Throughout the course of the experiment we ensured that there were three live tadpoles on the stimulus end of each aquarium each day. These tadpoles served as prey for the backswimmers. Placement of tadpoles on the stimulus end in the other treatments controlled for any effects related to the presence of prey tadpoles in the backswimmer treatment. We prepared the alarm cue stimulus by grinding a single tadpole with a mortar and pestle in 60 ml of distilled water. The resulting solution was filtered through a fine mesh net and 10 ml of the solution was added to each of the alarm cue treatment containers. Alarm cues were added to the aquaria three days per week. On all occasions the cues were introduced into the stimulus end of the tank.

Throughout the course of the experiment we fed the tadpoles ad libitum with ground alfalfa pellets. The aquaria were cleaned once per week. We monitored the experimental aquaria daily. All test animals that reached metamorphosis (Gosner stage 41) (Gosner, 1960) were removed from the aquaria and weighed.

For each aquarium we calculated the mean time tadpoles took to reach metamorphosis, and the mean mass at metamorphosis. Tank means were used in all statistical analyses. We used a multivariate analysis of variance (MANOVA) to examine the effects of the treatment conditions on metamorphic characteristics of the toads (Tabachnick and Fidell, 1989). After MANOVA, we used univariate analysis of variance (ANOVA) on each of the response variables (time taken to reach metamorphosis, size upon reaching metamorphosis, survival to metamorphosis) to assess which variables were responsible for significant main effects. Post hoc comparisons (Tukey tests) were performed to test for differences between means among the stimuli presented.

RESULTS

MANOVA revealed that there was an overall effect of the treatment conditions on the life history parameters that we measured (Table 1). A subsequent ANOVA showed that time taken to reach metamorphosis was significantly affected by the treatment condition. Tukey tests revealed that tadpoles metamorphosed faster in the presence of alarm cues than in the nonpredator control ($P = 0.029$). Similarly, tadpoles metamorphosed faster in the presence of predator cues than in the presence of nonpredator control cues ($P = 0.025$). There was no difference in time to metamorphosis between the predator and alarm cue treatments ($P > 0.95$). Neither mass at metamorphosis nor survival to metamorphosis was influenced by the treatment conditions (Figure 1). Percentage of survival (mean ± SE) to metamorphosis was 66.7 ± 8.3, 64.5 ± 10.5, and 60.4 ± 8.6 in the predator, nonpredator and alarm cue treatments, respectively.

TABLE 1. RESULTS OF MANOVA FOR OVERALL EFFECTS OF TREATMENT CONDITIONS ON METAMORPHIC TRAITS OF WESTERN TOADS AND ANOVAS FOR EACH RESPONSE VARIABLE[a]

	F	*df*	*P*
MANOVA	14.909	9, 17	<0.001
ANOVA			
Time	6.800	2, 9	0.016
Mass	1.764	2, 9	0.226
Survival	0.121	2, 9	0.888

[a]Response variables are time to reach metamorphosis (time), mass at metamorphosis (mass), and survival to metamorphosis (survival).

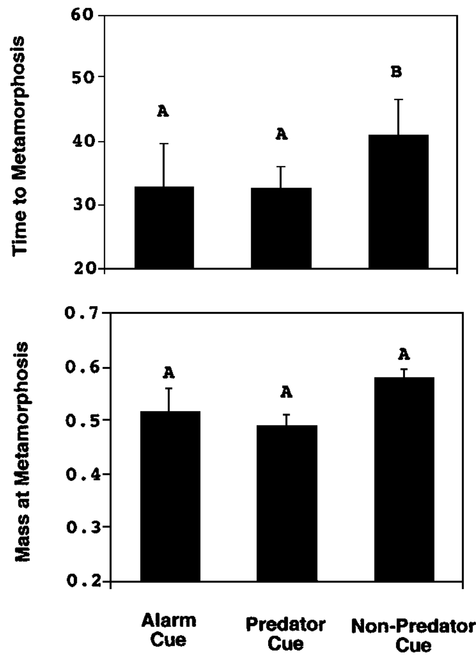

FIG. 1. Mean (+ SE) time to reach metamorphosis (days) and mean mass at metamorphosis (grams) for tadpoles exposed to chemical cues of injured conspecifics, cues of predatory backswimmers, and cues of nonpredatory water boatman. Different letters denote significant differences at $P < 0.05$, based on post-hoc Tukey tests.

DISCUSSION

The results of our study demonstrate that western toads alter characteristics of their life history in response to cues that indicate predation. Tadpoles decrease time taken to reach metamorphosis in the presence of predator cues and alarm cues when compared to nonpredator cues. By decreasing time taken to reach metamorphosis, toads likely benefit by reducing the time they are exposed to predation by aquatic predators. Nevertheless, this type of shift in life history may have potential costs. For example, early metamorphosis may expose toads to a higher level of terrestrial predation. Further experiments are needed to understand potential costs of early metamorphosis.

Kiesecker et al. (1996) showed that western toad tadpoles do not respond to visual cues of invertebrate predators (backswimmers and giant waterbugs), but do respond to chemical cues of the same predators. In this experiment we fed the backswimmers tadpoles in the experimental aquaria. Consequently, our

predator stimulus likely resulted in a complex stimulus that included both visual and chemical cues of the predator and injured prey cues. We know that both this complex predator stimulus and the injured prey (alarm) cues alone will induce the change in life history. It is important to stress that we do not know whether cues from the predator in the absence of alarm cues (bufotoxin) of the prey will induce this change in life history. The response to the predator stimulus that we observed may be a direct response to the alarm cues of injured prey and not a response to the predator per se.

Additional studies designed at examining the nature of the chemically induced life history changes are warranted. For example, future studies should manipulate the concentration and frequency of exposure to alarm cues in order to assess whether the change in life history is an all-or-nothing response or instead is a graded response that reflects the intensity of predation. Moreover, we should more closely examine the nature of the chemical cues that induce the changes, not only the chemistry of the stimulus but also its perception and the mechanism of response by the tadpoles. We know that alarm cues alone will induce a shift in life history. The response to the predator stimulus in our study may have been a response to the predator stimulus alone, it may have been a response to alarm cues released when the predator captured the prey, or alternatively it may be a response to alarm cues released in the predator's diet. Several behavioral studies indicate that prey species may only respond to chemical cues of a predator when the predator is fed a diet that contains conspecifics of the prey (e.g., Mathis and Smith, 1993; Wilson and Lefcort, 1993; Chivers et al., 1996). Similar results are known in studies of morphological defenses. For example, Stabell and Lwin (1997) showed that crucian carp (*Carassius carassius*) exhibit an adaptive change in body morphology in response to predators fed carp but not predators fed a different diet.

In our experiment the response of the toads was to decrease the time taken to reach metamorphosis. We found no evidence that tadpoles metamorphosed at a different size in the presence of the predator or alarm cues than in the presence of nonpredator cues. Van Buskirk (1988) and Wilbur and Fauth (1990) showed that American toad tadpoles decreased both the time taken to reach metamorphosis and the size at metamorphosis in response to dragonfly predators. In another study, Skelly and Werner (1990) found that American toads metamorphosed at a smaller size in the presence of larval dragonflies. They found no evidence that toad tadpoles reduced the time taken to reach metamorphosis.

Long-term shifts in life history may result from several factors. For example, numerous authors (e.g., Dodson and Havel, 1988; Skelly, 1992; Ball and Baker, 1996) have demonstrated that life history shifts may result as by-products of antipredator behavior. Specifically, time and energy devoted to antipredator responses have a cost in terms of a reduction in growth and/or development rate. Such reductions in growth and/or development rate could influence the timing

of life history switch points, for example, by either decreasing size at metamorphosis or increasing time to metamorphosis or both. Long-term shifts in life history may also represent facultative alterations in development rate, whereby animals increase their development rate while maintaining the same growth rate (e.g., Minchella and Loverde, 1981; Crowl and Covich, 1990; Wilbur and Fauth, 1990). This type of change would likely decrease the time taken to reach metamorphosis.

Changes in timing of metamorphosis or size at metamorphosis can occur even in the absence of a facultative increase in development rate or a decrease in growth and/or development rate associated with antipredator behavior. For example, if tadpoles have reached a plateau on their growth curve and if the conditions in the terrestrial environment are harsh or unpredictable, then in the absence of aquatic predators, tadpoles may not transform even in an aquatic environment that provides little or no growth opportunities. If this was the case, the costs of staying in the aquatic environment and not growing must outweigh costs in the terrestrial environment. It may be common to have temporary periods during which growth and/or survival is lower in the terrestrial environment than the aquatic environment. Such conditions could occur, for example, if the probability of desiccation is high or if terrestrial predators are concentrated at the edge of the water (DeVito et al., 1998).

In our experiment we documented that tadpoles were the same size at metamorphosis, but metamorphosed faster in the presence of the alarm and predator cues than in the presence of the nonpredator cues. We do not know whether tadpoles in the different treatments exhibited the same growth and/or development rate throughout the experiment. It is possible that differences in the timing of metamorphosis that we observed could result from a facultative increase in development rate. Alternatively, the tadpoles may have had the same growth and development rate but altered their timing of metamorphosis to reflect differences in costs and benefits of transforming. Future studies are needed to differentiate these possibilities.

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EFFECT OF DIMBOA, A HYDROXAMIC ACID FROM CEREALS, ON PEROXISOMAL AND MITOCHONDRIAL ENZYMES FROM APHIDS: EVIDENCE FOR THE PRESENCE OF PEROXISOMES IN APHIDS

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Abstract—2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), a hydroxamic acid involved in the resistance of cereals to aphids, was administered to adult individuals of the aphid *Sitobion avenae* in artificial diets. Effects on the cellular metabolism were inferred from the evaluation of several organelle marker enzymes. Catalase from peroxisomes and cytochrome *c* oxidase from mitochondria increased their activities about twofold when aphids were fed with 2 mM DIMBOA. The role of these enzymes in the metabolizing of xenobiotics by aphids is discussed. Biochemical and cytochemical evidences for the presence of peroxisomes in aphids are reported here for the first time.

Key Words—*Sitobion avenae*, Aphididae, aphids, hydroxamic acids, DIMBOA, catalase, cytochrome *c* oxidase, peroxisomes, mitochondria, xenobiotics.

INTRODUCTION

Aphids (Homoptera: Aphididae) are important crop pests that can cause serious agronomical losses by consuming nutrients from the host plant, injecting toxins,

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providing a medium for fungal development, and transmitting plant viruses. The presence of secondary metabolites in plants is of importance in their protection against herbivores such as aphids. Thus, phenolic compounds (Leszczynski et al., 1989), indole alkaloids (Zúñiga and Corcuera, 1986), and particularly hydroxamic acids (Hx) (Niemeyer, 1988; Argandoña et al., 1980; Bohidar et al., 1986; Thackray et al., 1990; Nicol et al., 1992; Niemeyer et al., 1993) have been described as defensive compounds in cereals. 2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) is the main Hx present in wheat extracts. This allelochemical produces several effects on aphids, including feeding deterrence, antibiosis, decreased reproduction, and decreased performance (Argandoña et al., 1983; Bohidar et al., 1986; Niemeyer et al., 1989; Givovich and Niemeyer, 1994, 1995). Little is known about the detoxification systems of plant allelochemicals, such as Hx, by aphids. A decrease in the activity of UDP-glucose transferases, involved in the detoxification of phenolic aglucones, has been reported in aphids fed with Hx (Leszczynski et al., 1992); an induction of glutathione-*S*-transferase activity has been shown in aphids from cereals with high concentrations of allelochemicals (Leszczynski et al., 1994); and an *in vitro* inactivation of aphid acetylcholinesterase by DIMBOA has also been reported (Cuevas and Niemeyer, 1993). In this study we evaluate the effect of DIMBOA on the activity of some subcellular organelle marker enzymes to determine their possible participation in Hx detoxification. We also report new evidence for the presence and function of peroxisomes in insects. These ubiquitous subcellular organelles participate in a variety of metabolic pathways, including detoxification of xenobiotics (Mannaerts and Van Veldhoven, 1993).

METHODS AND MATERIALS

Aphid Culture. One aphid clone collected and developed on wheat (*Triticum aestivum* cv. Paleta) under controlled conditions (20°C and 16L : 8D), and determined in our laboratory as *Sitobion avenae* with molecular markers (Figueroa et al., 1999), was used in this study. Previous to the experiments, the aphids were kept for at least four generations on oat (*Avena sativa* L. cv. Nahuén), an Hx-lacking cereal, to avoid the influence of Hx through maternal effects.

Treatment of Aphids with DIMBOA. Artificial diets containing 30% sucrose, amino acids, vitamins, and mineral salts, pH 5.5, were prepared as previously reported (Auclair, 1965; Argandoña et al., 1980). Fifty adult aphids were placed on a Parafilm sachet containing an artificial diet and encompassing a 60-mm-diam. Petri dish. The diets contained 0–8 mM DIMBOA, a concentration range comparable to that found in whole leaf extracts (Niemeyer et al., 1992) and in different plant compartments (Argandoña et al., 1987). DIMBOA was

prepared as solutions in dimethyl sulfoxide (DMSO), which were added to the artificial diets. The final concentration of DMSO was 0.001% v/v in all cases, including the control diets. Adult aphids were counted after 6–72 hr, and mortality was determined.

Homogenization and Subcellular Fractionation. At the end of the testing period, the living and effectively feeding aphids were transferred to 1.5-ml Eppendorf tubes containing ice-cold homogenization buffer [0.25 M sucrose, 3 mM imidazole, 1 mM EDTA, and 0.1% (v/v) ethanol, pH 7.4]. The aphids were homogenized with a plastic pellet pestle (Kontes) in ice. The homogenates were filtered through a single layer of cheesecloth, recovered in a fresh tube, and then centrifuged at 800*g* for 10 min to obtain a nuclear pellet and a postnuclear supernatant (PNS), according to conditions established previously (Santos et al., 1988). The PNS fraction was centrifuged at 105,000*g* for 35 min to obtain a pellet (P fraction), equivalent to the classic De Duve's MLP (M: heavy mitochondria; L: light mitochondria; P: microsomes) fraction, containing most of membranous organelles, and a supernatant (S fraction), containing mainly the cytosol. All these procedures were performed in the presence of a cocktail of protease inhibitors (0.5 mM each of chymostatin, leupetin, antipain, and pepstatin).

Density Gradient Fractionation. PNS fractions were subfractionated in a 56% Nycodenz continuous density gradient (density ranging from 1.00 to 1.30 g/ml) and used to separate and characterize animal peroxisomes and mitochondria. The PNS fraction was loaded over the gradient and subsequently centrifuged at 40,000 rpm for 49 min at 4°C in a Vti65 rotor. After centrifugation, fractions of 12 drops were collected from the bottom (heavier) to top (lighter) of the gradient solution, in preweighed tubes. The density was determined by measuring the refractive index of each fraction (Santos et al., 1994).

Enzyme Assays. Established procedures were used for the determination of activities of marker enzymes of subcellular organelles in homogenates, subcellular fractions, and density gradient fractions [catalase for peroxisomes, cytochrome *c* oxidase for mitochondria, *N*-acetyl-β-glucosaminidase (Naβgase) for lysosomes, and NADPH cytochrome *c* reductase for microsomes] (Santos et al., 1988). Proteins were determined by the Lowry method, with bovine serum albumin as standard (Lowry et al., 1951). The distribution of enzyme markers in fractionation experiments was calculated and represented as previously described (Bowers and de Duve 1967; De Duve and Baudhuin, 1966). Catalase latency was determined by measuring the activity of the enzyme in the presence/absence of detergent (Triton X-100, Sigma Co.).

Transmission Electron Microscopy and Catalase Cytochemistry. Aphids were fixed for 3 hr as a pellet with 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M PIPES buffer, pH 7.2, containing 0.2 M sucrose. The samples were postfixed in 1% osmium tetroxide, dehydrated, and embedded in Epon.

Ultrathin sections were further stained with uranyl acetate and lead citrate, and examined in a Phillips electron microscope (Figueroa et al., 1997).

Cytochemistry for catalase was performed by a modification of the alkaline diaminobenzidine method of Roels and Goldfischer (1979), exactly as described previously (Santos et al., 1994). Incubations in the absence of H_2O_2 and in the presence of the catalase inhibitor, 3-amino-1,2,4-triazole, served as controls.

Statistical Analysis. Comparison of means among different treatments were performed with the Mann-Whitney U test ($\alpha = 0.05$).

RESULTS AND DISCUSSION

The presence of four subcellular organelle marker enzymes in nontreated aphids was established (Table 1). To evaluate the effect of DIMBOA over the activity of these enzymes, aphids were fed with artificial diets containing different concentrations (0–8 mM) of the allelochemical during 48 hr. The most pronounced effect was observed with 2 mM DIMBOA (data not shown). At this concentration, cytochrome *c* oxidase and catalase increased their specific activities about twofold while Naβgase and NADPH cytochrome *c* reductase were not significantly altered (Figure 1). At higher concentrations of DIMBOA (>2 mM), a strong antifeeding effect and increased mortality were observed (data not shown), making these data less reliable. A range of feeding times (6–72 hr) with 2 mM DIMBOA was also tested. The effects on enzymes were highest at 48 hr of treatment (data not shown).

Cytochrome *c* oxidase is a protein of the inner membrane of mitochondria, which participates in the terminal complex of the mitochondrial respiratory chain (Van Kuilenburg et al., 1991). This enzyme has been the subject of several evolutive and physiological studies in insects such as flies, beetles, honeybees, cockroaches (De Bruijn, 1983; Hall and Smith, 1991; Azeredo-Espin et al., 1991; Martínez-González and Hegardt, 1994) and also aphids (Sunnucks and Hales, 1996).

TABLE 1. SPECIFIC ACTIVITIES OF SUBCELLULAR ORGANELLE MARKER ENZYMES IN HOMOGENATES OF APHID *Sitobion avenae*

Enzyme	Specific activity (mU/mg of protein)[a]
Catalase (peroxisomes)	0.64 ± 0.064 (14)
Cytochrome *c* oxidase (mitochondria)	9.2 ± 1.1 (7)
Naβgase (lysosomes)	11.7 ± 0.69 (8)
NADPH cytochrome *c* reductase (microsomes)	976 ± 84 (4)

[a]Mean ± standard error. Parentheses indicate the number of replicates.

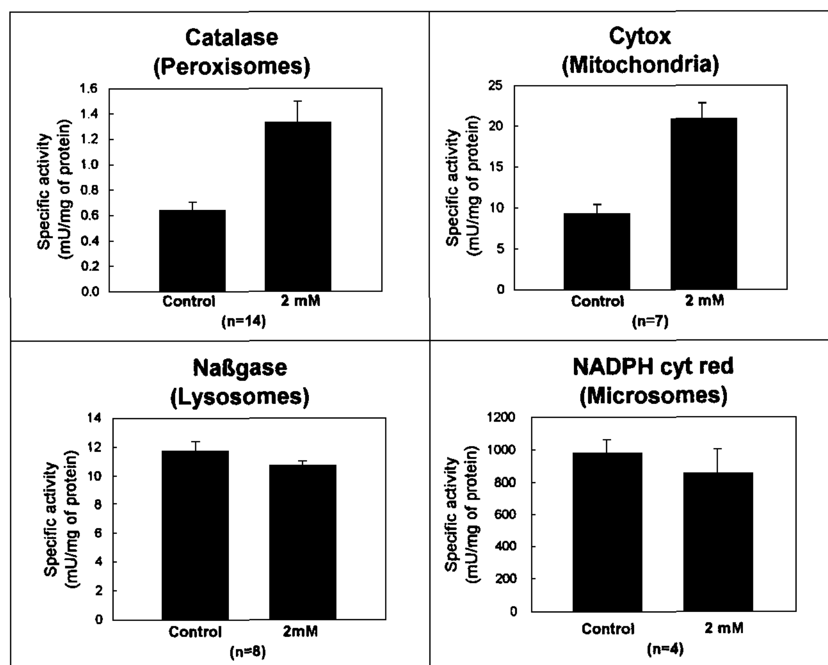

FIG. 1. Effect of administration of 2 mM DIMBOA in the diet of aphids during 48 hr, on organelle marker enzymes. The specific activities of four enzymes (cytox: cytochrome *c* oxidase; Naβgase: *N*-acetyl-β-glucosaminidase; NADPH cyt red: NADPH cytochrome c reductase) were measured in controls and treated (2 mM) aphid homogenates. Only the activities of catalase and cytochrome *c* oxidase were statistically different ($P < 0.05$) between treated and control aphids.

Catalase is a known peroxisomal marker enzyme, present in the matrix of peroxisomes from animals and plants (glyoxisomes) and also in the cytosol, that decomposes H_2O_2 into water and oxygen (De Duve and Baudhuin, 1966; Mannaerts and Van Veldhoven, 1993). Since no evidence was available on the presence of catalase or peroxisomes in aphids, the subcellular distribution of this enzyme was determined in *S. avenae* by cell fractionation in nontreated aphids. The sedimentable subcellular fraction (P fraction, containing most of the membranous organelles) was separated from the cytosolic fraction (S fraction, containing the cell cytosol) by high-speed centrifugation of a postnuclear supernatant extract. Figure 2a shows the distribution of catalase from nontreated aphids in the P and S fractions. Mitochondrial cytochrome *c* oxidase was used as an internal control for the fractionation experiments (Figure 2b). As expected, cytochrome *c* oxidase was found only in the P fraction, i.e., mitochondria. The

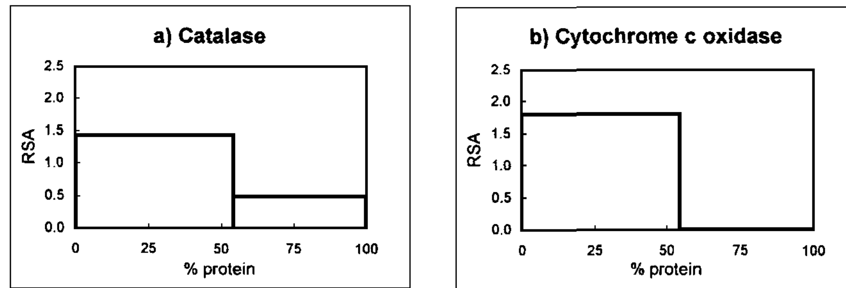

FIG. 2. Subcellular distribution of catalase (a) and cytochrome *c* oxidase (b) in a differential fractionation. A postnuclear supernatant was fractionated into an organellar pellet (P fraction, bar at left) and a supernatant (S fraction, bar at right). In the graphs the abscissa represents the cumulative protein content for each fraction as a percentage of the total protein of the homogenate. The ordinate represents the relative specific activity (RSA), i.e., percentage of activity of each enzyme in a given fraction of the homogenate relative to total activity over the percentage of homogenate protein in that fraction relative to total protein.

subcellular fractionation experiments showed the presence of catalase activity largely recovered in the organellar pellet (threefold more than in the S fraction), suggesting that this protein is contained in a sedimentable structure. Catalase latency in the P fraction was $73 \pm 1\%$, indicating that the majority of catalase in P is contained in a membrane organelle. These data are concordant with the proportion of relative specific activity found in the P fraction versus the S fraction. To isolate particles containing catalase, we used a Nycodenz density gradient subfractionation of aphid extracts. The particles containing catalase activity equilibrated in Nycodenz at 1.19 g/ml (Figure 3a), a density described for mammalian peroxisomes (Santos et al., 1994), and were clearly separated from mitochondria (1.14 g/ml) (Figure 3b). A morphological characterization of catalase-containing structures was undertaken by using the cytochemical diaminobenzidine method (Roels and Goldfischer, 1979) at the electron microscopy level (Figure 4). The diaminobenzidine reaction product in membrane-bound structures (Figure 4A) was not observed when the aphid sections were incubated in the presence of 3-amino-1,2,4-triazole, an inhibitor of catalase (Figure 4B and C). The presence of catalase in sedimentable particles showing an equilibrium density known for peroxisomes, and the structure of catalase-active particles observed under the electron microscope, indicate that aphids do contain peroxisomes and that catalase is a valid marker enzyme for peroxisomes in aphids. This organelle is known to participate in a variety of metabolic pathways such as lipid, purine, eicosanoid, phospholipid, and also xenobiotic metabolism (Mannearts and Van Vendhoven, 1993) and is an essential component in the cellular economy of several organ-

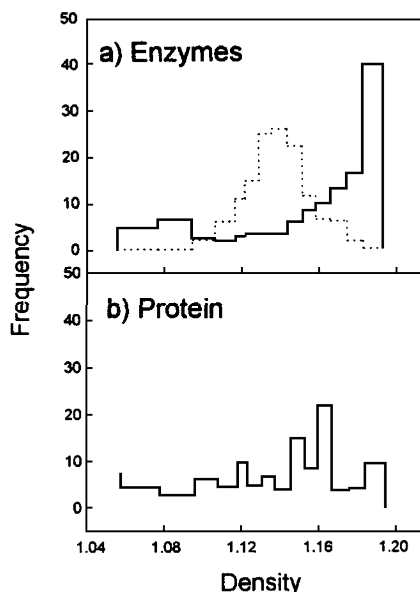

FIG. 3. Equilibrium density centrifugation of aphid postnuclear supernatants: (a) catalase (——), and cytochrome *c* oxidase (-----); and (b) proteins. The distribution pattern of catalase and cytochrome *c* oxidase corresponds to a representative fractionation experiment. For each distribution pattern, the ordinate represents the average frequency of the enzymes for each fraction. Frequency is plotted against density in a histogram form.

isms from yeasts to mammals (Lazarow and Fujiki, 1985). Although peroxisome functions in insect metabolism are still poorly understood, an organelle-bound catalase activity equilibrated in sucrose gradients at densities ranging between 1.19 and 1.22 g/ml and in Metrizamide at 1.15 and 1.21 g/ml has been reported recently in the honeybee midgut (Jiménez and Gilliam, 1996). This fact can also support the use of catalase as a peroxisomal marker enzyme in insects. In this case, the presence of peroxisomal catalase was associated with the metabolism of deleterious prooxidants from aerobic metabolism.

To investigate if the inducible catalase was peroxisomal and/or cytosolic, aphids treated with 2 mM DIMBOA for 48 hr were homogenized and fractionated by differential centrifugation. Figure 5a shows a significant twofold increment of sedimentable peroxisomal catalase activity in aphids treated with DIMBOA, while the effect on the cytosolic catalase activity was not significant (Figure 5b). The nonsignificant increment in the cytosolic catalase activity could be a consequence of the rupture of peroxisomes during the manipulations. The internal control of this fractionation experiment, the mitochondrial cytochrome

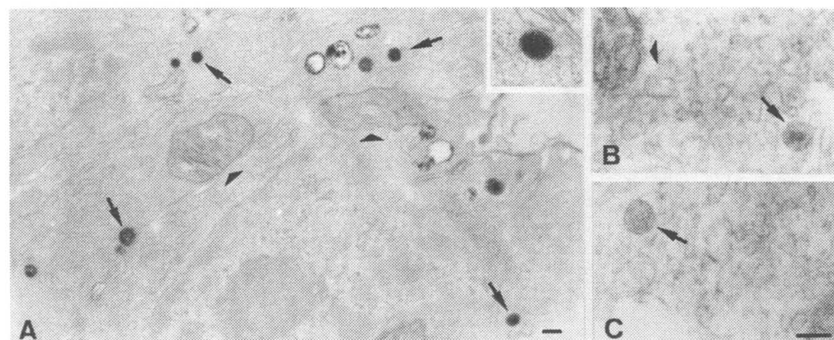

FIG. 4. Electron microscopy and catalase cytochemistry of epithelial tegumental cells of aphids. The aphids were fixed and subjected to conventional EM after cytochemistry for catalase in the absence (A) and in the presence of aminotriazole (B and C). The cytochemical reaction is clearly shown in thin sections, in which lead citrate staining has been omitted (A–C). A positive diaminobenzidine staining is concentrated inside of rather spherical granules (arrows in A–C), surrounded by a single membrane. A faint staining is also observed in the mitochondrial inner membrane (arrowheads). In the presence of aminotriazole, the reaction in the spherical granules is almost absent, showing that this reaction corresponds to catalase (B and C). Bar = 0.1 μm (A–C). Inset corresponds to a peroxisome at higher magnification.

c oxidase, was altered only in the pellet as expected (Figure 5c and d). Microsomal NADPH cytochrome *c* reductase and lysosomal Naβgase did not show any change.

It has been shown that DIMBOA is effectively ingested by aphids when they feed on artificial diets (Niemeyer et al., 1989; Givovich et al., 1992). The present results show an alteration of the oxidative cellular metabolism when aphids are treated with this plant secondary metabolite. Considering the lipophilic characteristics of DIMBOA, its incorporation into the cellular machinery through the cell membrane is likely. Within the cell, it would be metabolized by enzymes involved in defense against xenobiotics, such as glutathione-*S*-transferase, which has been shown to be inducible by the presence of high levels of cereal allelochemicals (Leszczynski et al., 1994). Their metabolism would produce oxidizable intermediates, which could induce the increase in the activity of enzymes involved in oxidative metabolism such as catalase and cytochrome *c* oxidase.

The increment in the activity of catalase and cytochrome *c* oxidase in aphids may represent another mechanism of insect resistance against plant secondary metabolites or insecticides, similar to the case of other xenobiotic metabolizing enzymes, i.e., cytochrome P-450s, glutathione-*S*-transferase, etc. (Mullin and

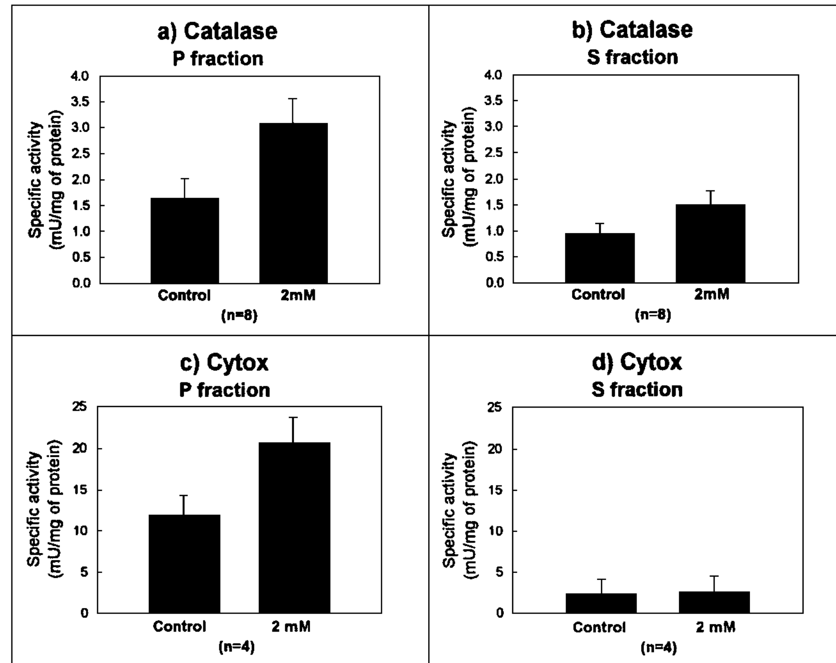

FIG. 5. Differential effect of DIMBOA on P and S subcellular fractions in aphids. (a) and (c) show the significant effects of 2 mM DIMBOA during 48 hr on specific activities of catalase ($P = 0.012$) and cytochrome *c* oxidase (cytox) ($P = 0.043$), respectively in the P fractions; (b) and (d) show lack of significant effects on both enzymatic specific activities in the S fractions ($P = 0.17$ and 0.773, respectively).

Scott, 1992). The evaluation of other enzymatic systems as well as the characterization of the metabolization route of DIMBOA will be essential to the understanding of the molecular mechanisms of action of cereal allelochemicals on aphids.

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CHEMICAL DEFENSE IN THE STINK BUG *Cosmopepla bimaculata*

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Abstract—Adult *Cosmopepla bimaculata* discharge a volatile secretion from paired ventral metathoracic glands (MTG) when disturbed. Collected volatiles were similar in both sexes and consisted of *n*-tridecane (67%), (*E*)-2-decenal (12%), (*E*)-2-decenyl acetate (12%), (*E*)-2-hexenal (3%), hexyl acetate (2%), *n*-dodecane (2%), a tridecene isomer (1%), and *n*-undecane, *n*-tetradecane, and *n*-pentadecane (all <1%). In addition, undisturbed males produced a novel insect compound, (*E*)-8-heneicosene, whose function is unknown. The MTG secretion emerges as an enlarging droplet, which is held in place by a cuticular projection and a pleural scent area consisting of specialized rough cuticle surrounding the gland opening. Insects can selectively discharge from either the right or left gland or both glands simultaneously, can control the amount of fluid ejected, and can resorb the ejected secretion droplet back into the gland reservoir. In feeding trials, killdeer (*Charadrius vociferus*), starlings (*Sturnus vulgaris*), robins (*Turdus migratorius*), and anole lizards (*Anolis carolinensis*) rejected or demonstrated aversion to feeding on the bugs. Furthermore, bugs that lacked the secretion were more susceptible to predation than bugs with secretion, suggesting that the secretion functions in defense against predators.

Key Words—*Cosmopepla bimaculata*, Pentatomidae, chemical defense, *n*-tridecane, (*E*)-2-decenal, (*E*)-2-decenyl acetate, (*E*)-2-hexenal, hexyl acetate, *n*-dodecane, *n*-undecane, *n*-tetradecane, pentadecane, (*E*)-8-heneicosene.

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INTRODUCTION

Stink bugs (Heteroptera: Pentatomidae) are well known for the odorous volatiles they emit when molested (Blum, 1981; Aldrich, 1988). These secretions typically contain mixtures of *n*-alkanes, alkenyl acetates, alkenols, and alkenals that primarily function in defense against predators (Staddon, 1979; Blum, 1981; Aldrich, 1988) or as attractants and sex pheromones (Aldrich, 1995, 1996; McBrien and Millar, 1999).

Cosmopepla bimaculata (Thomas) is a 5- to 7-mm long pentatomid found throughout most of the United States, northern Mexico, and southern Canada (Blatchley, 1926). In southern Illinois (USA), adults emerge from overwintering sites in early May and have been collected as late as mid-September in scattered groups of about 15, on plants in pastures and open areas (Esselbaugh, 1946; Fish and Alcock, 1973; McPherson, 1976). Adults are aposematic with red stripes on a shiny black background. In central Illinois, nymphs are also mildly aposematic with early instars being black and reddish orange and later instars exhibiting black patterns on a background that can range from cream or pale yellow to pink or yellow–orange (Decoursey and Esselbaugh, 1962). Both adults and nymphs emit an odorous secretion when squeezed, suggesting that the secretion functions in chemical defense against predators. In this paper we identify the chemical components and glandular source of the secretion, investigate the stimuli that elicit secretion expulsion, examine the palatability of *C. bimaculata* against various predators, and test the hypothesis that the secretion acts to deter predators.

METHODS AND MATERIALS

Insect Collection and Care

Adult *C. bimaculata* were collected from obedient plant, *Physostegia virginiana* (L.) Benth. (Lamiaceae), and lousewort, *Pedicularis canadensis* L. (Scrophulariaceae), in and near Normal, Illinois, from mid-June through early October. Thereafter, the bugs were maintained until needed in clear plastic containers (5 liters) at 25°C under a 14L : 10D photoperiod, and fed daily with fresh green beans, *Phaseolus vulgaris* L.

Chemical Analysis

Volatile Collection and Analysis. Thirty *C. bimaculata* adults were separated by sex with the aid of a microscope. Individuals were then squeezed and quickly placed into individual Hewlett-Packard autosampler vials (glass, 12 × 32 mm, sealed with an 11-mm Teflon-lined septum and aluminum crimp cap). For

controls, undisturbed adult males and females were also individually isolated in autosampler vials. Sampling of released volatiles was by solid-phase microextraction (SPME). The fiber coating was 100 μm poly(dimethylsiloxane). SPME equipment was obtained from Supelco (Bellefonte, Pennsylvania). To sample, we inserted the sheath of the SPME through the septum, then extended the fiber, exposing it to the volatiles for 30–45 min. Volatiles were then injected into a GC for analysis (Hewlett-Packard 5890 Series II, equipped with an HP Chemstation, a split/splitless injector run in splitless mode, and a flame ionization detector). Duration of SPME injection was 30 sec, and the purge valve was opened after 30 sec. The DB-5 capillary column (J & W Scientific, Folsom, California) was 30 m $\times$ 0.25 mm ID and had a 1.0-μm film thickness. The GC oven was held at 50°C for 1 min, then raised to 250°C at 10°C/min, and then held at 250°C for 6 min. Inlet temperature was 200°C and the detector temperature was 250°C. Relative amounts of compounds emitted by the bugs were calculated from SPME-GC peak areas, after correcting for differences in fiber sensitivity (Bartelt, 1997).

Mass spectra were obtained on a Hewlett-Packard 5973 MSD, with sample introduction through a DB-1 capillary column (15 m $\times$ 0.25 mm ID with 0.1-μm film thickness, temperature held at 50°C for 1 min, then raised to 250°C at 10°C/min, and then held at 250°C for 6 min). To analyze larger volatiles [i.e., (*E*)-8-heneicosene DMDS derivative] the final temperature was set at 300°C.

Because electron impact mass spectra make interpretation of double bond positions in hydrocarbons unreliable, the heneicosene found in adult males was derivatized with dimethyl disulfide (DMDS) so that the double-bond location could be determined. Five dead males were extracted by covering them with 2.5 ml of hexane and crushing with a glass rod. To purify the hydrocarbons, the extract was filtered, reduced in volume under N_2 (to concentrate before derivitization), and then applied to a 3- $\times$ 0.5-cm column of silica gel. The hydrocarbons were eluted with hexane. The DMDS adduct was then synthesized and the mass spectrum of this derivative was interpreted according to the procedure of Carlson et al. (1989).

Chemicals Used. We purchased *n*-undecane, *n*-dodecane, *n*-tridecane, *n*-tetradecane, and *n*-pentadecane (all 99%) from Sigma Chemical Co. (St. Louis, Missouri); (*E*)-2-hexenal (99%) and (*E*)-2-hexenol (96%) from Aldrich Chemical Co. (Milwaukee, Wisconsin); and (*E*)-2-decenal from Lancaster Synthesis (Windham, New Hampshire). (*E*)-8- and (*Z*)-8-Heneicosenes were available from a previous investigation (Bartelt and Jackson, 1984).

We synthesized two esters that were not obtained commercially, (*E*)-2-decenyl acetate and hexyl acetate. To make the former, (*E*)-2-decen-1-ol was first prepared by reducing (*E*)-2-decenal with $LiAlH_4$. The aldehyde (3 g in 10 ml dry ether) was added dropwise to 10 ml of a stirred 1 M solution of $LiAlH_4$ in ether under N_2 at 0°C. The temperature was not allowed to exceed 15°C. When the addition was complete, the solution was warmed to room temperature and

stirred for 2 hr. Then 2 g of $MgSO_4 \cdot 7H_2O$ was added to decompose the excess reagent and to release the alcohol. The solution was filtered, and the solvent was evaporated in vacuo. (*E*)-2-Decenyl acetate was prepared by allowing a mixture of (*E*)-2-decen-1-ol (1.1 g), acetic anhydride (1.1 g), triethylamine (1.5 ml), and 4-(dimethylamino)pyridine (0.075 g) to stand for 24 hr at room temperature. Ether and 2 N HCl were added, and the organic phase was washed with saturated $NaHCO_3$, dried over $MgSO_4$, and the solvent evaporated in vacuo (Höfle et al., 1978). The resulting product, (*E*)-2-decenyl acetate, was >97% pure by GC. We used the same procedure to prepare hexyl acetate (>95% pure product) from hexanol. All synthetic compounds gave satisfactory mass spectra.

Behavioral Response Towards Threats

We used a 3-mm-diam. wooden dowel to examine the response of *C. bimaculata* to threats. Ten wild adults were tested in the field on a warm sunny day (33–35°C) by (1) moving the tip of the dowel at a rate of approximately 2 cm/sec several times to within 0.5 cm of the insect, then (2) poking the insect with the dowel, and finally, (3) grabbing the insect and gently rolling it between the thumb and forefinger. The resulting behaviors were recorded. In addition, secretion discharge in nymphs and adults and the deterrent quality of the secretion were tested by placing bugs on our tongues and pressing them against our palates.

Predator Feeding Trials

Killdeer. A 1-week-old *Charadrius vociferus* L. was reared in the laboratory on a diet of arthropods, earthworms, snails, fish, and fruit. At about four weeks of age, when the bird could feed by itself and discriminate among prey (i.e., began to prefer some prey items over others), it was tested with the following method: The bird was given a control third-instar *Acheta domesticus* (L.) cricket to demonstrate hunger. Then, *C. bimaculata* individuals were sequentially offered until the bird no longer consumed the bugs. When this occurred, the bird was then offered another control cricket to verify that the bird was still hungry. If the control was eaten, then another bug was offered. Uneaten prey were removed 4 min after being placed in the cage with the bird. The experiment ended when the bird continued to eat the controls, but repeatedly rejected the bugs.

European Starling. A 3-week-old *Sturnus vulgaris* L. was reared in the laboratory on a diet of arthropods, fruit, and cat food. When the bird showed strong food discrimination patterns (i.e., began to prefer some foods over others), it was tested. The feeding trials were conducted in the same way as for *C. vociferus*.

American Robin. A 1-week-old *Turdus migratorius* L. was reared in the laboratory on a diet of arthropods, fruit, and cat food. When the bird was able to discriminate between food items and feed without assistance, it was tested as above, except that dewinged adult house flies (*Musca domestica* L.) were used

as controls and, after six bugs were consumed, flies and bugs were alternated as prey. We recorded number of prey attacked, number of prey consumed, and time to attack (the time from introduction of the insect until the bird pecked at or lowered its head toward the insect).

Green Anoles. Four adult *Anolis carolinensis* Voigt, obtained from a local pet shop, were maintained for two weeks at 26°C with surplus food (second to fourth instar *A. domesticus*) and water. The anoles were isolated and starved for two days prior to testing. Each anole was tested individually in its home container by introducing a series of adult *C. bimaculata*. Small, second to fourth instar *A. domesticus* crickets served as control prey.

To begin each test, an anole was initially offered a cricket to verify hunger. Next, *C. bimaculata* adults were sequentially offered until the anole refused to attack them. This two-step procedure was repeated until the anole continually rejected *C. bimaculata*, yet continually ate controls. Following this first trial, the anoles were starved for 48 hr and then tested again using the same procedure.

Test of Secretion Function

To test the hypothesis that the metathoracic gland secretion of adult *C. bimaculata* serves a defensive function, we compared the response of lizard predators to milked vs. unmilked bugs. Five adult green anoles were each offered a series of adult *C. bimaculata* that had been milked of their secretion by repeatedly squeezing and washing them under water. Five other adult anoles were offered unmilked bugs. Each anole was tested in its home cage and was given a series of *C. bimaculata* until it failed to consume three successive bugs. Prey were offered at about 4-min intervals, and each of the 10 trials was completed within 10–55 min. We noted aversive behaviors and the total number of prey eaten for each anole.

Source of Volatiles

We examined *C. bimaculata* adults and nymphs under a dissecting microscope to determine the presence and location of external gland orifices. We then tested four locations as the possible source of the bugs' secretion: tip of beak, anus, metathoracic gland (MTG), and dorsal abdominal gland (DAG). Seven adult males and seven adult females were chilled to 10°C. Five of each sex were glued ventral side up onto cardstock. The remaining four adults were dewinged with small scissors and glued ventral side down. All bugs were then allowed to warm to room temperature, viewed under a dissecting microscope, and stroked, poked, or pinched with tweezers to elicit expulsion of the secretion. Slivers of filter paper (ca. 3.0 mm^2) were then placed on either the MTG opening, dorsal abdomen, beak, or anus to absorb any ejected fluids. We conducted similar experiments with fourth and fifth instars.

RESULTS

Chemical Analyses

Both male and female *C. bimaculata* produced a blend of at least 11 different compounds when agitated. Nine of the compounds yielded good-quality mass spectra that were tentatively identified by matching to library spectra. These identifications were confirmed when commercial or synthetic standards gave identical mass spectra and GC retention times (Figure 1A, Table 1). The relative compositions of the identified volatiles were not significantly different between males and females (two-sample t test). Pooled over both sexes ($N = 18$), the mean percentages and their standard errors for the compounds were: n-tridecane, 67.4% ± 1.4; (E)-2-decenal, 12.2% ± 1.5; (E)-2-decenyl acetate, 11.5% ±

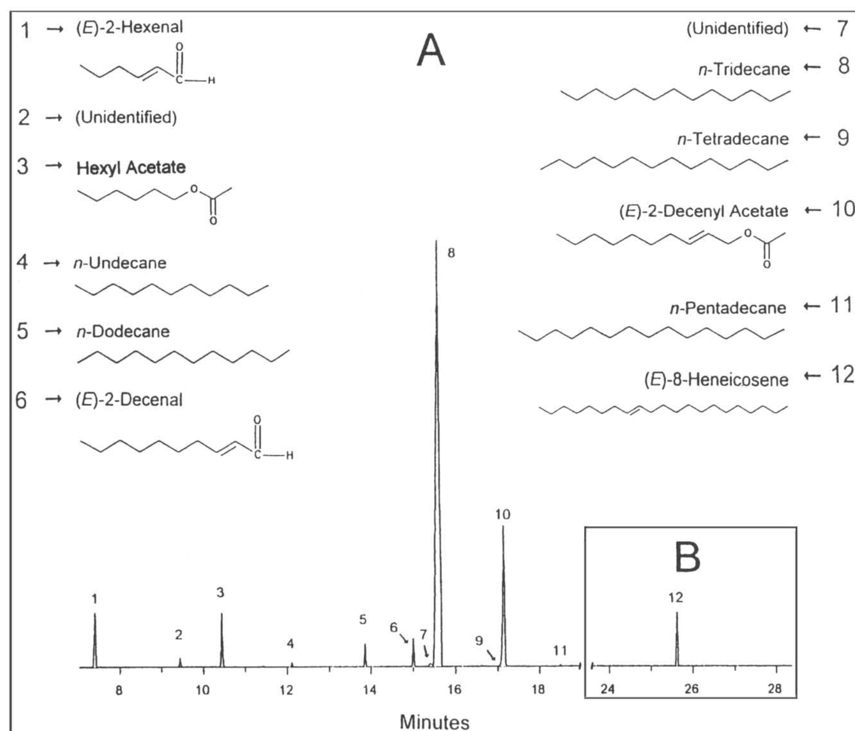

FIG. 1. (A) Gas chromatogram of volatile emissions from disturbed adult female *Cosmopepla bimaculata*. (B) Volatile emission from a non-disturbed adult male *C. bimaculata*. Peak numbers correspond to those in Table 1.

TABLE 1. CHEMICALS IDENTIFIED IN *C. bimaculata* SECRETIONS

GC Peak	Identification	Major MS ions[a] (percentages)
1	(*E*)-2-hexenal	98 (M+, 26), 83 (68), 69 (83), 57 (49), 55 (90), 42 (60), 41 (100), 39 (69)
2	(Unidentified)	112 (M+, 15), 97 (1), 83 (100), 69 (2), 57 (19), 55 (61), 39 (5)
3	Hexyl Acetate	144 (M+, NOT SEEN), 101 (2), 84 (22), 73 (10), 69 (19), 61 (24), 56 (49), 55 (24), 43 (100)
4	*n*-Undecane	156 (M+, 10), 85 (38), 71 (61), 57 (100), 43 (83), 41 (43)
5	*n*-Dodecane	170 (M+, 8), 85 (46), 71 (74), 57 (100), 43 (88), 41 (55)
6	(*E*)-2-Decenal	154 (M+, 0.4), 83 (61), 70 (88), 69 (54), 57 (64), 55 (92), 43 (100), 41 (100), 39 (67)
7	?-Tridecene	182 (M+, 8), 111 (19), 97 (46), 83 (63), 69 (78), 55 (100), 43 (86), 41 (92)
8	*n*-Tridecane	184 (M+, 7), 85 (48), 71 (77), 57 (100), 43 (99), 41 (83)
9	*n*-Tetradecane	198 (M+, 8), 85 (52), 71 (76), 57 (100), 43 (86), 41 (46)
10	(*E*)-2-Decenyl Acetate	198 (M+, 0.1), 156 (14), 110 (22), 96 (26), 95 (19), 82 (24), 81 (26), 68 (21), 67 (28), 55 (29), 54 (34), 43 (100), 41 (25)
11	*n*-Pentadecane	212 (M+, 6), 85 (46), 71 (69), 57 (100), 43 (70), 41 (34)
12[b]	(*E*)-8-Heneicosene	294 (M+, 21), 111 (50), 97 (90), 83 (97), 71 (46), 70 (51), 69 (88), 57 (84), 56 (50), 55 (100), 43 (88), 41 (70)

[a]Major MS ions (m/z), in order of decreasing m/z.
[b]Compound detected only from some male samples (see text).

1.0; (*E*)-2-hexenal, 3.3% ± 0.6; hexyl acetate, 2.2% ± 1.0; *n*-dodecane, 1.7% ± 0.1; ?-tridecene, 1.4% ± 0.8; *n*-undecane, 0.2% ± 0.02; *n*-tetradecane, 0.1% ± 0.02; *n*-pentadecane, 0.07% ± 0.01.

As suggested by the standard errors (above), the proportions of individual secretion components varied from bug to bug, particularly with some of the lower-molecular-weight compounds. For example, in some samples, compound 2 was more abundant than compound 1 or 3, whereas in other samples, compound 2 could not be detected. Additional compounds not depicted in Figure 1 occasionally appeared in the glandular secretions of *C. bimaculata*. For example, we tentatively identified small concentrations of (*E*)-2-octenal in some adult male and female secretions.

No volatiles were collected from nonagitated adult female *C. bimaculata*

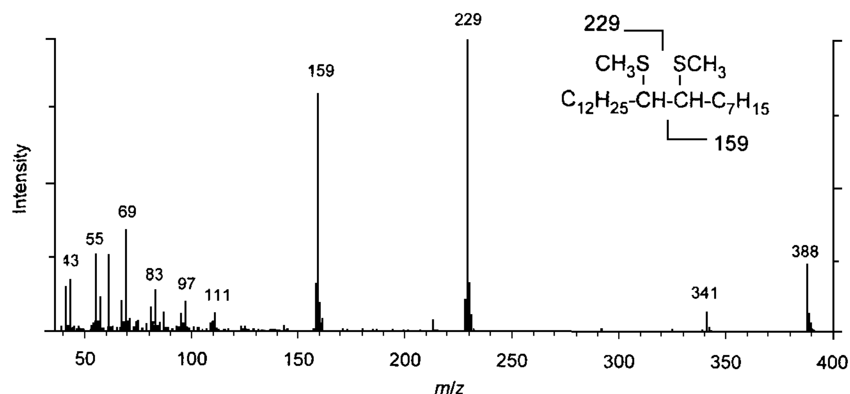

FIG. 2. Mass spectrum of the DMDS adduct of the insect-derived (*E*)-8-heneicosene, illustrating key peaks with m/z of 159 and 229.

but three of four undisturbed adult males (collected in early August 1997) produced one compound (Figure 1B). This compound was tentatively identified as a heneicosene isomer by its mass spectrum (Table 1). The double bond position (= 8) was then determined from the mass spectrum of the DMDS adduct, which had major fragment peaks at m/z 159 and 229 (Figure 2). The standard 8-heneicosene and its DMDS adduct gave corroborative mass spectra. Our synthetic standard 8-heneicosene contained both *E* (ca. 25%) and *Z* (ca. 75%) isomers, and their linear temperature programmed retention indexes, relative to *n*-alkanes, were 2078 and 2073, respectively, on the DB-1 column. The insect-derived compound matched the *E* isomer in GC retention, and this was confirmed by co-injection of insect-derived and standard samples. The amount of (*E*)-8-heneicosene was not determined because in October, when we analyzed the defensive secretions quantitatively, field-collected adults showed no traces of (*E*)-8-heneicosene.

Behavioral Response Towards Threats

Adult *C. bimaculata* showed little defensive behavior when initially approached with a 20-cm-long dowel. Only when the dowel was about 5 mm away from the bugs, or touching them, did they react by slowly crawling away from the dowel to the opposite side of the leaf or stem. A continued, gentle prodding of the bugs caused them to move away, hide within a node or flower, drop to the ground, or fly away, but failed to cause them to expel perceivable volatiles. The bugs secreted only after they were picked up and roughly handled. Other bugs secreted after being pinched on their antennae or legs. Adults and fourth and fifth instars that were placed in human mouths immediately secreted when mildly

TABLE 2. ADULT *Cosmopepla bimaculata* AND CONTROL PREY (CRICKETS OR HOUSE FLIES) CONSUMED BY BIRDS DURING ONE FEEDING TRIAL

	C. bimaculata		Controls	
Predator	Offered (*N*)	Eaten (*N*)	Offered (*N*)	Eaten (*N*)
Killdeer	5	1	6	6
Starling	4	0	5	5
Robin	12	8	12	12

squeezed between the tongue and palate, producing an instantaneous burning sensation and chemical taste that lingered for up to 20 min. This was followed by a slight localized numbness of the tongue, which lasted 1–2 hr. We were surprised at the intensity of the burning sensation and taste delivered by even small fourth instars. Newly hatched first instars produced no secretion or taste when placed on the tongue and gently squeezed. However, when chewed, the burning and taste characteristic of the secretion appeared, although less intense than in older nymphs.

Predator Feeding Trials

The killdeer readily attacked and consumed the first bug offered, which expelled its secretion, producing a strong odor. The bird then rejected the next four bugs, and would: (1) bob its head toward its prey as if to attack and then quickly withdraw without touching the prey; (2) step rapidly around the bug while pecking at the cage bottom near the bug; or (3) completely ignore the bug (this behavior was exhibited toward the last bug offered). All six cricket controls were immediately consumed by the bird, and, on three occasions, the bird stepped over a bug to get to a cricket (Table 2).

The starling showed strong aversion towards adult *C. bimaculata*. The bird quickly attacked the first bug offered, but immediately ejected the still-living bug out of its beak. The odor of the defensive secretion was strong. Five seconds later, the bird repeated these behaviors, then ignored all subsequent bugs, but eagerly consumed all crickets (Table 2).

The robin demonstrated mild aversion towards adult *C. bimaculata*. It attacked 11 of 12 bugs offered and consumed eight of these. In contrast, it attacked and consumed all 12 houseflies offered (Table 2). However, the robin took significantly longer to attack *C. bimaculata* than house flies (two sample t test, $df = 10$, $0.05 > P > 0.025$) (Table 3). During the trial, *T. migratorius* showed aversive behavior toward *C. bimaculata* such as ejecting bugs out of the beak and running toward newly introduced bugs and then turning away. Near the end of the trial, the bird was standing in the middle of the cage and was given a

TABLE 3. TIME (MEAN ± SE) FOR *T. migratorius* TO ATTACK *C. bimaculata* VS. HOUSE FLIES

Predator	Time to attack (sec)	
	C. bimaculata	*M. domestica*
Robin	40.6 ± 19.4 (N = 11)	2.3 ± 0.3 (N = 12)

choice between two bugs positioned in opposite corners of the cage and a house fly that was introduced in a third corner of the cage. The bird quickly ran toward one bug, turned and ran toward the other bug, and then turned and ran toward the control fly, which was promptly seized and consumed. The bird continued to ignore the two bugs crawling about the cage, but ate two additional flies.

One of the four anoles, upon sampling its first bug, vigorously threw the bug out of its mouth, wiped its snout on the bottom of the cage, then ignored all subsequent bugs, but readily consumed all controls offered. The other three anoles readily attacked and consumed the first bug offered, but became more hesitant with the second or third bug, and eventually ignored the fourth (Table 4). When attacked, the bugs emitted their strong, characteristic odor. When these same anoles were retested two days later, three refused to attack, one ate one *C. bimaculata*, yet all anoles eagerly attacked and consumed controls.

Test of Secretion

Five anoles that were offered *C. bimaculata* adults that lacked MTG secretion ate 5, 6, 6, 6, and 9 bugs, respectively ($\overline{X} \pm$ SE = 6.4 ± 0.68). Five other anoles that were given fully charged bugs at 1, 1, 2, 2, and 3 bugs, respectively ($\overline{X} \pm$ SE = 1.8 ± 0.37). The means are significantly different (t test, $P < 0.001$), suggesting that milked bugs were more palatable to anoles than unmilked bugs.

During these tests, anoles demonstrated aversion behaviors toward the

TABLE 4. ADULT *C. bimaculata* AND SECOND–THIRD INSTAR CRICKETS CONSUMED BY LIZARDS DURING TWO TRIALS SEPARATED BY TWO DAYS

Anole	Day 1		Day 3	
	C. bimaculata eaten (N)	*A. domesticus* eaten (N)	*C. bimaculata* eaten (N)	*A. domesticus* eaten (N)
1	3	5	0	3
2	3	3	0	3
3	0	4	0	4
4	3	2	1	4

charged bugs, but not the milked bugs. These aversive behaviors included spitting out the bug, backing away from the bug, wiping the head and mouth upon substrate, excessive mouthing before swallowing, and ignoring the bugs upon their introduction.

Source of Volatiles

All nymphal stages of *C. bimaculata* appeared to have three pairs of orifices on the dorsal abdomen corresponding to the DAGs of other Heteroptera. When fourth or fifth instars were squeezed by the antennae or legs, liquid emerged from the DAG orifices and spread over the surrounding cuticle. When absorbed onto slivers of paper, this liquid smelled similar to the adult defensive secretion. Nymphal stages lacked ventral MTG openings, and no liquid appeared on the venters of nymphs when molested. Occasionally liquids appeared at the mouth or anus of the disturbed nymphs and adults, but these liquids were odorless, suggesting that these structures were not the source of the defensive secretion.

Adults lacked discernible DAGs, and no liquid appeared on adult dorsums when adults were squeezed. However, adults of both sexes had paired MTG openings. Each orifice was surrounded by an extensive pleural scent area of dull, rough cuticle, which contrasted with the glossy, smooth cuticle, covering the remainder of the insect.

Various stimuli elicited secretion discharge from the MTGs. When adults (glued upside down to a piece of cardstock) were calm, they secreted only when strongly squeezed on an appendage or on the body. However, once agitated (by prior squeezing), adults would discharge when lightly stroked with a fine paintbrush on the eyes, antennae, thorax, or abdomen.

The insects could selectively secrete from either the left or right MTG and could control the volume of discharged secretion. When agitated adults were stroked on the right side, only the right gland discharged. Pressure applied to the head or abdomen caused both glands to discharge. When lightly stimulated, only a small droplet of secretion appeared at the gland orifice. When strongly stimulated (crushing an appendage) droplets larger than the insect's compound eyes emerged from the MTG orifices.

Discharge of secretion was usually accompanied by a telescoping of the abdomen into the rigid thoracic box. Secretion did not spray out, but emerged as an enlarging droplet. As the liquid flowed out of the MTG orifice, it formed a spherical droplet that was held in place by a cuticular projection at the gland orifice. Typically, droplets sat for 2–3 sec and then were drawn back into the insect's body. The pleural scent area appears to cause the droplet to bead up, and thus serves to hold the droplet in place. However, when agitated adults touched a leg to the droplet, it immediately wetted the cuticle of the leg and thorax by spreading over it.

DISCUSSION

Chemistry of C. bimaculata *Secretion*

Disturbed adult male and female *C. bimaculata* secrete a similar blend of at least 11 volatile compounds (two aldehydes, two esters, five alkanes, and two unidentified compounds). Approximately 92% of the secretion consisted of only three components: *n*-tridecane (68%), (*E*)-2-decenal (12%), and (*E*)-2-decenyl acetate (12%). The metathoracic gland (MTG) secretions of male and female *C. bimaculata* were nearly identical, other than the presence of (*E*)-8-heneicosene found in some males (which may or may not originate from the MTG).

Overall, the secretion of *C. bimaculata* contains typical pentatomid MTG compounds (Staddon et al., 1987; Aldrich, 1988, 1995; Nagnan et al., 1994; McBrien and Millar, 1999) and is strikingly similar to *Erthesina fullo*, *Lincus malevolus*, and *L. spurcus* (Kou et al., 1989; Nagnan et al., 1994). Of the nine components identified in the defensive secretion of both male and female adult *C. bimaculata*, seven [(*E*)-2-hexenal, (*E*)-2-decenal, and the C_{11}–C_{15} hydrocarbons] are common in the secretions of terrestrial arthropods. For example, (*E*)-2-hexenal exists in the secretions of cockroaches (Farine et al., 1997), beetles (Tschinkel, 1975), ants (Crewe et al., 1972), and many true bugs (Aldrich, 1988; Leal et al., 1994), and (*E*)-2-decenal has been identified in cockroaches (Wallbank and Waterhouse, 1970), beetles (Tschinkel, 1975), and various heteropterans (Farine et al., 1992; Aldrich et al., 1993). Likewise, *n*-tridecane occurs in thrips (Suzuki et al., 1989), ants (Bellas and Hölldobler, 1985), mites (Kuwahara et al., 1991), moths (Severson et al., 1991), and numerous Heteroptera (Farine et al., 1992; Aldrich et al., 1993). In contrast, two of the compounds in the defensive secretion of adult *C. bimaculata* [hexyl acetate and (*E*)-2-decenyl acetate] are generally found only within the Heteroptera (Blum, 1981; Surender et al., 1987; Aldrich, 1988; Gunawardena and Bandumathie, 1993; Leal et al., 1994; Millar et al., 1997; Millar and Rice, 1998).

The (*E*)-8-heneicosene isolated from males early in the season is interesting because, to our knowledge, this is the first *E* configuration for a natural insect alkene. However, similarly structured C_{21} cuticular hydrocarbon isomers [e.g., (Z)-10-heneicosene] have been found in the Diptera and Coleoptera (Bartelt et al., 1986; Peschke and Metzler, 1986).

Function of Secretion

Our results suggest that the secretion of *C. bimaculata* functions in antipredator defense because: (1) The secretion is ejected in response to disturbance. (2) Some secretion components [(*E*)-2-hexenal, *n*-undecane, *n*-dodecane, and *n*-tridecane] are known toxins, irritants, or repellents (Blum, 1981; Whitman et al., 1990). (3) Bugs lacking the secretion were more susceptible to predation

than bugs with secretion. (4) The secretion composition is similar in both sexes, suggesting that the secretion is not used as a sexual pheromone. (5) The bugs exhibit traits commonly associated with chemically defended insects (Pasteels et al., 1983; Guilford, 1990; Vulinec, 1990) such as conspicuous coloration, gregariousness, diurnal activity, and poor locomotory capability (e.g., when approached, they do not readily fly and can easily be caught with the fingers). (6) All the predators we tested developed some sort of food aversion towards the bugs: The starling refused to consume a single *C. bimaculata*; the killdeer ate one, but rejected all others; the anoles ate them initially, but rejected them during the second trial; and the robin exhibited strong attack hesitancy. In addition, these predators displayed various aversive, conflict, or displacement behaviors (Gustavson, 1977), such as ejecting bugs from their mouths, wiping their mouths on the floor as if to clean off irritating chemicals, reversing or interrupting predation in mid-attack, or running rapidly about the cage pecking near, but not at, the bug. These behaviors strongly imply that the bugs were distasteful.

It is common for different predators to respond differently to the same prey (Whitman, 1988; Whitman et al., 1990), and in our study, each predator species responded differently to *C. bimaculata*. All of our predators were naive (had never encountered *C. bimaculata* before), and all eagerly attacked the first bug offered. However, subsequent predatory behaviors varied according to predator species; each was deterred, but apparently via a different mechanism. The starling rejected *C. bimaculata* almost immediately upon sampling, suggesting that rejection in this predator occurred via an immediate, stimulus–response reaction mediated by secretion–stimulation of peripheral chemosensilla in or near the mouth. Hence, with this predator, the secretion was strong enough to elicit ejection from the mouth before the bug was killed. This is significant because it implies individual selection for defensive attributes (Wiklund and Järvi, 1982; Sillén-Tullberg and Bryant, 1983; Engen et al., 1986; Guilford, 1990). In contrast, three of four anoles demonstrated strong feeding aversions only after the first day and only after consuming several bugs (Table 4). This suggests that aversion in some anoles is mediated in part by an internal physiological response, such as toxicosis, which occurs after the bugs are swallowed and requires time to develop. This type of predator response implies kin or group selection (Fisher, 1958; Harvey and Greenwood, 1978; Guilford, 1990) as the driving force for the evolution of chemical defense in *C. bimaculata*. Finally, the results with the robin suggest that *C. bimaculata* is only partially deterrent to this predator and would be eaten when alternative prey were absent.

It is not known how the various secretion components interact. However, bioassays conducted by Gunawardena and Herath (1991) have shown that two common components of pentatomid secretions [(*E*)-2-hexenal and *n*-tridecane] were more effective as repellents to insects when combined than when individually tested. Furthermore, they found that other *n*-alkanes, when combined with

(*E*)-2-hexenal, were not as effective deterrents towards other insects as *n*-tridecane. Hence, *n*-tridecane appears to be the optimal *n*-alkane to work synergistically with (*E*)-2-hexenal to repel insects. The other secretion components of the multicomponent blend of *C. bimaculata* may likewise function in an additive way.

In addition to its defensive role, the secretion of *C. bimaculata* may serve other functions. In the field, *C. bimaculata* are often highly clumped, which suggests that they may possess aggregation or sexual pheromones, as do other pentatomids (Aldrich, 1995, 1996; McBrien and Millar, 1999). Conversely, the secretion could function as an alarm pheromone. Indeed, adult *C. bimaculata* immediately dropped off plants at the approach of fingers contaminated with secretion. Conversely, the secretion could act against entomopathogenic fungi. In bioassays, the hemipteran exocrine products hexyl acetate, (*E*)-2-decenal, and tridecane have all exhibited fungicidal activity (Surender et al., 1987; Sosa-Gomez et al., 1997).

Secretion Ejection

Theory predicts and observations confirm that insects are frugal when using their valuable defensive secretions (Wallace and Blum, 1969; Pasteels et al., 1984; Whitman et al., 1990). For some insects, regeneration of lost defensive stores may require weeks or months, leaving the prey more susceptible to predation (Fescemyer and Mumma, 1983; Carrel, 1984; Whitman et al., 1992). Hence, arthropods are generally reluctant to discharge, often waiting until stimuli indicate a clear and immediate danger (Eisner et al., 1976; Blum, 1981; Whitman et al., 1991). In our trials, *C. bimaculata* adults did not readily expel their secretion: no discharge occurred in response to visual threats or to mild tactile stimulation. Insects discharged only after they were rolled in the fingers or squeezed. There may be an additional benefit in not discharging prematurely: secreting in a predator's mouth (which contains a high density of chemosensilla) might be a more effective predator deterrent than secreting in response to the approach or initial investigative touches by a potential predator.

That the bugs expelled secretion when squeezed by the antennae or legs and could selectively emit from just one gland shows that discharge is not passive, but under neural–muscular control; external pressure is not required to force the secretion out. Indeed, pentatomids are known to possess complex musculature facilitating secretion discharge from internal reservoirs to the outer surface of the insect (Staddon, 1979). The morphology of the plural scent area allows adults to expel and hold droplets of secretion, which, if danger passes, can be pulled back into the gland reservoir and later reused. Alternatively, when further threatened, a bug can quickly coat itself with a rapidly volatilizing protective film of secretion, by touching a leg to the droplet, causing it to spread immediately over the glossy, waxy cuticle.

Function of (E)*-8-Heneicosene*

(*E*)-8-Heneicosene was found in only males, suggesting its possible role as a male sex pheromone. Male stink bugs are well known to produce attractive pheromones (Aldrich, 1995; McBrien and Millar, 1999). The hypothesis that (*E*)-8-heneicosene serves a pheromonal role is supported by an anecdotal observation: adults overwinter, and mate and oviposit during the late spring to late-summer. We observed widespread copulations in early August when (*E*)-8-heneicosene was detected among field collected males. However, no copulations were observed in the same population consisting of newly eclosed adults collected in early October when there was no detection of (*E*)-8-heneicosene from field collected males. For now, both the glandular source and the function of (*E*)-8-heneicosene remains uncertain, requiring further research to establish its role in the life history of this insect.

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ALLELOCHEMICALS ISOLATED FROM TISSUES OF THE INVASIVE WEED GARLIC MUSTARD (*Alliaria petiolata*)[1]

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Abstract—Garlic mustard (*Alliaria petiolata*) is a naturalized Eurasian species that has invaded woodlands and degraded habitats in the eastern United States and Canada. Several phytotoxic hydrolysis products of glucosinolates, principally allyl isothiocyanate (AITC) and benzyl isothiocyanate (BzITC), were isolated from dichloromethane extracts of garlic mustard tissues. AITC and BzITC were much more phytotoxic to wheat (*Triticum aestivum*) than their respective parent glucosinolates sinigrin and glucotropaeolin. However, garden cress (*Lepidium sativum*) growth was inhibited to a greater degree by glucotropaeolin than BzITC, possibly due to conversion to BzITC by endogenous myrosinase. Sinigrin and glucotropaeolin were not detected in leaf/stem tissues harvested at the initiation of flowering, but were present in leaves and stems harvested in the autumn. Sinigrin levels in roots were similar for both sampling dates, but autumn-harvested roots contained glucotropaeolin at levels over three times higher than spring-harvested roots. The dominance of garlic mustard in forest ecosystems may be attributable in part to release of these phytotoxins, especially from root tissues.

Key Words—Garlic mustard, *Alliaria petiolata*, Brassicaceae, glucosinolates, allelopathy, phytotoxins, allyl isothiocyanate, benzyl isothiocyanate, sinigrin, glucotropaeolin.

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[1] Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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INTRODUCTION

Garlic mustard [*Alliaria petiolata* (Bieb) Cavara & Grande (Brassicaceae)] is an herbaceous biennial that has invaded, and now dominates, much of the hardwood forest understory in the eastern and midwestern United States and southeastern Canada (Cavers et al., 1979; Nuzzo, 1991, 1993, 1998). It is displacing native flora, and it is unlikely that its elimination from heavily infested areas is possible (Anderson et al., 1996). Populations of native understory plants have been found to decline in areas with a heavy infestation of garlic mustard, which can be as high as 20,000 seedlings/m^2 (Trimbur, 1973; Yost et al., 1991). Groundcover by native ephemerals declined as cover by garlic mustard increased (Nuzzo, 1998). Recent studies have shown that garlic mustard may also pose a threat to organisms other than higher plants, as Porter (1994) reported that adults of the endangered West Virginia white butterfly [*Pieris virginiana* (W. H. Edwards)], which normally feed on several *Dentaria* spp. (Brassicaceae), preferentially laid their eggs on garlic mustard plants. This occurs even though the plant appears to be moderately toxic to the developing larvae (Haribal and Renwick, 1998).

Glucosinolates and/or their degradation products appear to be primarily responsible for the pesticidal activity of species in the Brassicaceae (syn. Cruciferae) (Brown et al., 1991; Grossman, 1993; Brown and Morra, 1995; Mayton et al., 1996; Mancini et al., 1997; Vaughn and Boydston, 1997). Glucosinolates are a class of glucose- and sulfur-containing organic anions whose biologically active degradation products are produced when plant cells are ruptured and the glucosinolates, which are present in vacuoles, are hydrolyzed by the enzyme myrosinase (β-thioglucosidase glucohydrolase; EC 3.2.3.1) (VanEtten and Tookey, 1983). These metabolites include substituted isothiocyanates, nitriles, thiocyanates, and oxazolidinethiones, which vary depending on the side-chain substitution, cell pH, and cell iron concentration (Cole, 1976; Daxenbichler and VanEtten, 1977; Fenwick et al., 1983; Uda et al., 1986; Chew, 1988). Some of these degradation products have been found to be potent phytotoxins (Wolf et al., 1984; Oleszek, 1987; Bialy et al., 1990; Yamane et al., 1992a,b; Brinker and Spencer, 1993; Brown and Morra, 1995; Vaughn et al., 1996; Vaughn and Boydston, 1997; Vaughn and Berhow, 1998). The leaves and seeds of garlic mustard have been previously shown to contain a high percentage of glucosinolates (up to 3% of fresh weight in seeds), with the predominant glucosinolate being allyl glucosinolate (sinigrin) (Nielsen et al., 1979; Larsen et al., 1983; Daxenbichler et al., 1991).

It is presently unclear whether the dominance of garlic mustard in forest groundlayers is due to competition, allelopathy, or both (Randall, 1996), although a recent report by McCarthy and Hanson (1998) discounted allelopathy as a primary mechanism. To further elucidate if allelopathy plays a role in garlic mustard dominance, we present results from a bioassay-guided isolation and identification of phytotoxins from garlic mustard plants.

METHODS AND MATERIALS

Spectroscopy. Gas chromatography–mass spectrometry (GC-MS) was performed on a Hewlett-Packard (HP) 6890 GC system attached to a HP 5972A Mass Selective Detector. Columns used were fused silica HP-5MS capillaries (0.25-μm film thickness, 30 m $\times$ 0.25 mm ID). The GC operating parameters were as follows: splitless injection mode; temperature programmed from 40° to 315°C at 5°C/min with a 2-min initial and a 10-min final temperature hold; He carrier gas flow rate at 1.1 ml/min, with the injector temperature set at 250°C. Spectra were compared with known standards or by computer with the Wiley/NBS Mass Spectral Registry (McLafferty and Stauffer, 1989).

Extract Preparation. Garlic mustard tissues (100 g samples) were sequentially extracted using a Soxhlet apparatus with hexane, CH_2Cl_2, and MeOH, and concentrated by rotoevaporation at low (20°C for hexane and CH_2Cl_2 extracts, 50°C for MeOH extracts) water bath temperatures, preventing possible loss of volatile extraction products. A water extract was obtained by soaking the solvent-extracted tissues in 250 ml of distilled water overnight in a refrigerator at 2°C, after which the marc was washed with two additional 250-ml aliquots, and the extracts lyophilized. Compounds in the crude CH_2Cl_2 extract, subsequently found to be active in the bioassays, were separated on a lipophilic Sephadex LH-20 (Supelco, Inc., Bellefonte, Pennsylvania) column into three separate fractions using 100% $CHCl_3$; 50% $CHCl_3$/50% MeOH; and 100% MeOH as solvents.

Seedling Radicle Elongation Bioassay. Wheat (*Triticum aestivum* L., Cardinal) and cress (*Lepidium sativum* L. Curly Cress, Brassicaceae) seeds were used in routine bioassays of extracts. Wheat and cress seeds were surface sterilized with 0.5% (w/v) commercial chlorine bleach for 15 min, rinsed with sterile distilled water (SDW) twice and subsequently soaked with additional SDW for 2 hr. Seeds were wrapped in sterile paper towels saturated with water and incubated overnight in darkness at 25°C. All crude extracts were assayed by adding extracts to autoclaved water agar in 9.0-cm plastic Petri dishes at the concentration of 1 mg extract/ml agar after the agar had cooled to ~ 40°C. Column fractions from crude extracts were assayed at concentrations of 0.1 and 0.5 mg extract/ml agar. After the agar had solidified and all solvent had evaporated from the agar, six germinated seedlings of each bioassay species per plate were placed on the agar in the Petri dishes. Dishes were incubated in darkness at 25°C on 45° slants for 24–48 hr, then evaluated for inhibition of radicle growth.

Allyl isothiocyanate (AITC), benzyl isothiocyanate (BzITC), and sinigrin standards were obtained from a commercial source (Sigma, St. Louis, Missouri). The glucotropaeolin standard used in bioassays was extracted and purified to greater than 98% from cress seeds by the method of Thies (1988). Solutions of the isothiocyanates (dissolved in acetone) and intact glucosinolates (dissolved in water) were added to cooling water agar to give final concentrations of 0,

10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} M (controls contained acetone only). Plates were sealed with Parafilm (American National Can, Neenah, Wisconsin) to prevent volatilization of the isothiocyanates. Radicle lengths (five plates of six seedlings each of wheat and cress) were measured after 48 hr of incubation, and I_{50} values (the amount of each compound required to reduce radicle elongation by 50%) were estimated from the intercept of 50% of the control with a best fit line of the data using nonlinear regression analysis (SlideWrite Plus, Advanced Graphics Software, Inc., Carlsbad, California).

Glucosinolate Analysis. Glucosinolate concentrations were determined from leaf/stem and root tissues of garlic mustard plants that were harvested on May 7, 1998 (at initiation of flowering), and on October 30, 1998, from plants growing in a oak–hickory (*Quercus–Carya*) forest in Peoria, Illinois. The analytical method employed was a modification of a high-performance liquid chromatography (HPLC) method developed by Betz and Fox (1994). In brief, 5 g of freeze-dried plant material was added to 200 ml boiling 70% (v/v) MeOH with stirring for 15 min, and then cooled and filtered through Whatman No. 2 filter paper. The marc was washed twice with 50 ml aliquots of 70% MeOH. The resulting extract was concentrated to 5–10 ml by rotoevaporation and was diluted to 25 ml to form a working solution. Glucosinolates were purified from the extracts through the use of disposable solid-phase extraction (SPE) columns (Sep-Pak tC_{18}, Waters Corp., Milford, Massachusetts). Each column was preconditioned with 5 ml 100% MeOH, followed by 5 ml 0.005 M tetrabutylammonium hydrogen sulfate (THS; Sigma). Five milliliters of the working solution was added to the column, and the column was subsequently washed with 5 ml of 0.005 M THS to remove unwanted compounds. Glucosinolates were eluted from the SPE column with 2 ml MeOH/THS (55 : 45), and run on a Shimadzu 6A HPLC system using a C_{18} column (250 mm × 4.6 mm; RP-18, 5μ; Licrosorb, Alltech, Deerfield, Illinois). The glucosinolate peaks were detected with a Shimadzu SPD-M6A photodiode array detector set at 237 nm. The initial mobile phase conditions were 12% methanol–88% aqueous 0.005 M THS at a flow rate of 1 ml/min. The binary gradient was developed to 70% methanol–30% aqueous 0.005 M THS for 20 min, and held at these conditions for an additional 15 min. Concentrations of sinigrin and glucotropaeolin were calculated from standard curves developed for both compounds.

RESULTS AND DISCUSSION

Identification of Phytotoxins from Extracted Tissues. The crude CH_2Cl_2 extract strongly inhibited cress and wheat radicle elongation. However, the methanol extract was only slightly inhibitory, while the hexane and water extracts had no effect. Fractionation of the crude CH_2Cl_2 extract on the

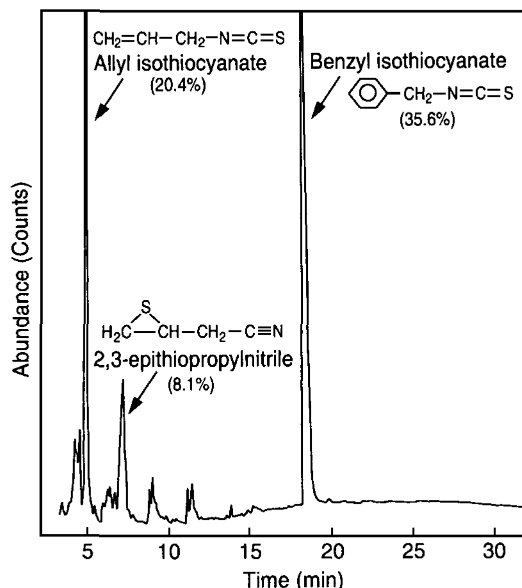

FIG. 1. Gas chromatograph and identification of major compounds in active 100% CH_2Cl_2 fraction from garlic mustard plants.

Sephadex LH-20 column yielded only one fraction (100% $CHCl_3$) that was highly inhibitory to radicle elongation at 0.5 mg extract/ml agar and that was also very active at the 0.1 mg extract/ml concentration. This fraction contained three major peaks as determined by GC-MS (Figure 1), which were identified by comparison with published mass spectra (Kjær, 1963; Spencer and Daxenbichler, 1980) and comparison with spectra obtained from commercial standards as AITC, BzITC and 2,3-epithiopropylnitrile. After further fractionation on a Sephadex LH-20 column using CH_2Cl_2, separate fractions containing AITC and BzITC were bioassayed as active at 0.1 mg extract/ml agar. A fraction containing primarily 2,3-epithiopropylnitrile was not active at 0.5 mg extract/ml agar. Prominent diagnostic mass spectral ions and their relative intensities for the isolated AITC and BzITC are as follows: AITC: EI-MS [*m*/*z* (%)]: 99 (M^+, 88), 72 (34), 45 (12), 41 (100). BzITC: EI-MS [*m*/*z* (%)]: 149 (M^+, 18), 91 (100), 65 (15), 51 (6).

Toxicity of Isothiocyanates and Parent Glucosinolates. The calculated I_{50}s for wheat and cress, respectively, were as follows: for sinigrin, $>1 \times 10^{-3}$ and 4.4×10^{-4} M; for glucotropaeolin, $>1 \times 10^{-3}$ and 6.6×10^{-4} M; for AITC, 4.1×10^{-5} and 8.5×10^{-4} M; and for BzITC, 5.2×10^{-5} and 1.9×10^{-4} M (Figure 2). The higher toxicities of sinigrin and glucotropaeolin to cress may be due

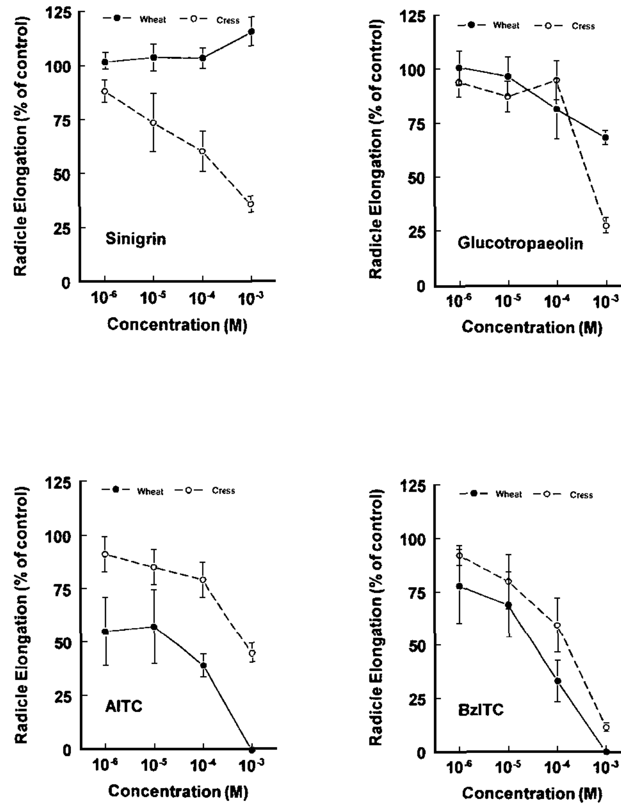

FIG. 2. Inhibition of wheat and cress radicle elongation by sinigrin, glucotropaeolin, AITC and BzITC. Bars represent ± 1 SE.

to hydrolysis of glucosinolates in the cress seedlings by endogenous myrosinase (which is absent in wheat), producing AITC and BzITC. Although both AITC and BzITC were quite phytotoxic in these bioassays, AITC in particular appears to be even more potent as a volatile, because Vaughn and Boydston (1997) reported the complete inhibition of seed germination and growth of seven plant species by this compound at a level of 1 ppm. Perhaps the bioassay that we employed in this study is less sensitive in determining toxicities of compounds with little or no water solubility such as AITC and BzITC.

Glucosinolate Content of Garlic Mustard Tissues. There were significant differences in glucosinolate levels in spring-harvested garlic mustard tissues compared to those in autumn-harvested tissues (Table 1). Both sinigrin and glucotropaeolin were below detection limits (<1 μg/mg freeze-dried tissue) for

TABLE 1. SINIGRIN AND GLUCOTROPAEOLIN CONTENT OF GARLIC MUSTARD PLANTS FROM SPRING AND AUTUMN HARVEST

	Freeze-dried tissue (μg/mg)[a]	
Tissue	Sinigrin	Glucotropaeolin
Spring leaf/stem	0.0c	0.0c
Spring root	3.5a	15.3b
Autumn leaf/stem	2.8ab	0.0c
Autumn root	2.8ab	50.2a

[a]Means within a column followed by the same letter are not different at $P = 0.05$ according to Fisher's protected LSD test.

leaves and stems from spring-harvested plants, although autumn-harvested plants had detectable levels of both compounds. Root tissues from both spring and autumn had similar levels of sinigrin, but glucotropaeolin levels were more than three times greater in autumn-harvested than spring-harvested roots.

McCarthy and Hanson (1998) suggested that allelopathy was not the primary method of interference with garlic mustard and that future research should focus on its competitiveness. As these authors point out, garlic mustard is a highly competitive plant that is self-compatible, sets large amounts of seed, germinates under a wide range of environmental conditions, and grows vigorously at near-freezing temperatures in the late autumn and early spring when leaves are absent from deciduous trees. These researchers reported that a dilution series of aqueous extracts from garlic mustard roots and shoots did not reduce germination of the seeds of several plants, including rye (*Secale cereale* L.), hairy vetch (*Vicia villosa* Roth), and lettuce (*Lactuca sativa* L.), with only the strongest dilution tested, 1 : 10, being inhibitory to radish (*Raphanus sativus* L.). In their research, McCarthy and Hanson (1998) prepared extracts by macerating garlic mustard root and shoot tissue in a vegetable juicer and diluting the mixture with distilled water to form a dilution series. Presumably, most of the glucosinolates present in these tissue would be quickly hydrolyzed by endogenous myrosinase to form breakdown products during the extraction process. It is unlikely that AITC and BzITC, which are practically insoluble in water, would remain solubilized, or even be present, in these aqueous extracts. The slight activity seen by these researchers against radish seeds may be similar to the results found in this study against cress seeds, as residual intact glucosinolates could be converted to phytotoxic hydrolysis products by endogenous myrosinase present in both species.

On several occasions when walking in areas heavily infested with garlic mustard plants, the senior author could detect the distinctive odor of AITC emanating from the plants. We were able to collect measurable amounts of both AITC and BzITC once via solid-phase microextraction (SPME) collection from

plants in vivo, but unfortunately, we have not been able to repeat this observation (data not presented). AITC, which presumably has a very low odor threshold for humans, has been detected only on relatively warm days in the autumn, but curiously not in either the spring or the summer. Perhaps the higher glucosinolate levels we found in the autumn-harvested plants were the cause of the detectable levels of breakdown products we observed.

As both AITC and BzITC have been found to be inhibitory to fungi (Patil et al., 1973; Holley and Jones, 1985; Gamliel and Stapleton, 1993), another potential role of these compounds, in addition to direct allelopathic effects on neighboring plants, is the inhibition of mycorrhizal fungi. 4-Hydroxybenzyl isothiocyanate, the major hydrolysis product of the glucosinolate glucosinalbin, was found to be the predominant compound in roots of wild mustard [*Brassica kaber* (DC.) Wheeler], which inhibited germination of the vesicular-arbuscular mycorrhizal fungus *Glomus etunicatum* (Shreiner and Koide, 1993a,b). Garlic mustard, like other members of the Brassicaceae, is nonmycorrhizal, while the majority of competing plant species present in the oak–hickory understory are mycorrhizal (Harley, 1969). Further study of volatiles released by garlic mustard plants in vivo, and their effect on mycorrhizal associations, is warranted.

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GEOGRAPHIC VARIATION IN SEX PHEROMONE BLEND OF *Hemileuca electra* FROM SOUTHERN CALIFORNIA

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Abstract—Differences were found in the pheromonally mediated mate location systems of two subspecies of *Hemileuca electra*, *H. electra electra* (Hee) and *H. e. mojavensis* (Hem), from southern California. Hem female pheromone gland extracts contained eight times as much (10*E*,12*Z*)-hexadeca-10,12-dienal (*E*10,*Z*12–16 : Ald) and half as much hexadecyl acetate (16 : Ac) as Hee extracts. Relative amounts of the other major component of the pheromone blends, (10*E*,12*Z*)-hexadeca-10,12-dien-1-ol (*E*10,*Z*12–16 : OH) did not differ between the two subspecies. In coupled gas chromatography-electroantennogram studies, responses of male antennae to 1 : 1 : 1 mixtures of the three principal components (*E*10,*Z*12–16 : Ac, *E*10,*Z*12–16 : OH, *E*10,*Z*12–16 : Ald) also differed, with Hem antennae producing significantly larger responses to *E*10,*Z*12–16 : Ald and significantly smaller responses to 10*E*,12*Z*–16 : Ac than Hee. In field trials, male Hem were attracted to Hem females in preference to Hee females. Males of a second species, *H. burnsi*, which is sympatric with Hem but not Hee, also were attracted to females of Hee transported to their range. Field tests of blends of synthesized pheromone components confirmed that male Hem preferred *E*10,*Z*12–16 : Ald ratios of 10–100% of the major component, *E*10,*Z*12–16 : Ac, whereas males of Hee and *H. burnsi* responded optimally to ratios of 0.3–1% *E*10,*Z*12–16 : Ald to *E*10,*Z*12–16 : Ac. 16 : Ac added to lures increased attraction of Hee but not Hem males. The data presented are consistent with reproductive character displacement, whereby the Hem subspecies has modified its pheromone-based mating system to reduce interference from sympatric *H. burnsi*.

Key Words—*Hemileuca electra electra*, *Hemileuca electra mojavensis*, *Hemileuca burnsi*, (10*E*,12*Z*)-hexadeca-10,12-dien-1-yl acetate, (10*E*,12*Z*)-hexadeca-10,12-dien-1-ol, (10*E*,12*Z*)-hexadeca-10,12-dienal, hexadecyl acetate, sex pheromone, reproductive character displacement.

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INTRODUCTION

Hemileuca electra Wright (Lepidoptera: Saturniidae) is one of the most common saturniid moths found in the coastal sage habitat of southern California. It also can be abundant on the lower elevations of mountain slopes throughout the Mojave desert. In areas where the larval host, *Eriogonum fasciculatum* (G. Bentham) is abundant, males frequently can be seen flying erratically over low vegetation during the morning to early afternoon hours in late September through early November. The adults do not possess functional mouthparts and cannot feed, and as a result, adult life-spans are short (<1 week). Consequently, virtually all adult behaviors are directed towards mate location or oviposition. Females call in the morning hours when males are actively seeking mates. Mating lasts approximately 30–60 min, following which females begin ovipositing at dusk. In addition to actively searching for mates, males also passively seek mates by perching on shrubs with the antennae elevated into the wind (J.S.M., personal observation). When at rest, the antennae are held underneath the wings such that pheromone cannot reach them.

The range of *H. electra* extends from central Arizona, north to southern Utah, westward throughout the Mojave desert, and southward into central Baja California (Tuskes and McElfresh, 1995). Two subspecies, *H. electra electra* Wright (Hee) and *H. electra mojavensis* Tuskes & McElfresh (Hem), are found in California. Hee ranges along the coast of southern California in an area bounded to the north by the Transverse mountain ranges and to the east by the Peninsular ranges. Hem is found near mountain ranges throughout the Mojave desert to the north of the Transverse mountain ranges. Gene flow between the two subspecies, Hee and Hem, is attenuated by the mountain ranges but can occur through the Cajon, San Gorgonio, and other passes, and there is documented evidence of hybrid populations (Tuskes and McElfresh, 1995).

The range of Hem overlaps extensively with that of *Hemileuca burnsi* Watson in the Mojave desert (Tuskes, 1984; Tuskes et al., 1996). The two species often are found together along the lower elevations of the northern slopes of the San Gabriel and San Bernardino mountains. Field studies with live moths have demonstrated that females of the coastal subspecies *H. electra electra* Wright are attractive to males of the Mojave desert subspecies, *H. electra mojavensis* Tuskes & McElfresh, and also to the congeneric Mojave desert species *H. burnsi* (Tuskes, 1984; Tuskes et al., 1996). The two species are quite different in appearance and host preferences, and matings between *H. electra* and *H. burnsi* do not produce viable offspring (J.S.M., unpublished data).

The objectives of this study were: (1) to determine whether there is significant variation in the pheromone-mediated mate location systems of the two *H. electra* subspecies, and (2) to determine the role of pheromone blends in

the prezygotic reproductive isolation of the sympatric congeners, *H. electra mojavensis* and *H. burnsi*.

The results of this study support, but cannot prove, a case for reproductive character displacement. The pheromone blend produced by female *H. electra mojavensis*, the subspecies sympatric with *H. burnsi*, is significantly different than the blend produced by the allopatric subspecies *H. electra electra*. Furthermore, the latter blend is attractive to both *H. electra mojavensis* and to *H. burnsi*.

METHODS AND MATERIALS

Insects. The *H. e. electra* used in this study were obtained from several locations in southern California: (1) 4 mi east of Aguanga, Riverside County, (2) Lake Hodges, San Diego County, and (3) Lake Skinner, Riverside County. The *H. e. mojavensis* were collected at two locations: (1) Ord Mountains east of Hesperia, San Bernardino County, California, and (2) Rock Corral, San Bernardino County, California. Last instars were collected in late February through May on the larval host, *Eriogonum fasciculatum*, and reared on living *E. fasciculatum* in 3.2-mm mesh hardware cloth cages (60 cm diam. × 45 cm high) covered with fiberglass screening. Pupae were retrieved from the rearing cages and kept in screen-covered 40-liter glass aquaria outdoors where they would experience natural light and temperature. Adults emerged in late September through early November. Moths were collected in the morning hours before they had an opportunity to mate and were kept in 6- × 8-cm screen cages or glassine envelopes inside sealed plastic bags with a damp towel at 10–15°C for one to two weeks. Virgin females were used for field studies and preparation of pheromone extracts, while males were used for electroantennographic studies.

Extraction of Sex Pheromone Glands. Sex pheromone glands were removed from virgin calling females 1–4 days old. The gland was extruded by applying gentle pressure to the abdomen near the tip, and the terminal abdominal segments were excised with small scissors into a 0.25-ml conical glass vial insert containing ~30 μl pentane. Glands were extracted 20 min, and then the solvent was transferred to a clean insert, with two rinses with one drop of pentane. Extracts were concentrated by passive evaporation as needed prior to analysis. If not used immediately, extracts were stored at −20°C.

Gas Chromatography. GC analyses were conducted with Hewlett-Packard 5890A or 5890 Series II gas chromatographs (H-P, Palo Alto, California), with helium carrier gas, in splitless mode. Columns (J&W Scientific, Folsom, California) and programs used to analyze composite extracts from multiple females were DB-Wax (30 m × 0.32 mm ID, 0.25 μm film, 100°C at 0 min, rising 10°C/min to 250°C, and held for 45 min) and DB-5 (30 m × 0.32 mm ID,

0.25 μm film, 100°C at 0 min, rising 10°C/min to 240°C and then 25°C/min to 275°C, and held for 45 min).

Coupled Gas Chromatography–Electroantennography (GC-EAD). Aliquots of single female extracts, composite female extracts, and synthetic standards were analyzed by splitless coupled GC-EAD, in an H-P 5890 series II GC equipped with a DB-5 column (30 m × 0.32 mm ID, 0.25 μm film; J&W Scientific), programmed from 100°C at 0 or 1 min, then 10 or 15°C/min to 275°C, and held for 25 min. The column effluent was split equally with a press-fit Y-connector (J&W Scientific) into two branches of 0.25-mm uncoated fused silica tubing, with one branch going to the flame ionization detector (FID) and the other being directed through a heated transfer line (275°C) to a 1-cm-diam. glass stimulus delivery tube. The capillary effluent was diluted with humidified air (400 ml/min) and passed over the male moth antennal preparation. The moth antenna was suspended between glass capillary electrodes filled with insect physiological saline (modified Locke's saline, 7.5 g NaCl, 0.21 g $CaCl_2$, 0.35 g KCl, 0.20 g $NaHCO_3$ in 1 liter distilled water) (Humason, 1972), with electrical contact to the custom-built multistage amplifier made with AgCl-coated silver wires. The amplifier and FID outputs were simultaneously recorded on a matched pair of H-P 3394 recording integrators.

Male antennae were removed by using fine forceps to firmly hold the scape and pull the antenna free of the head, without crushing the scape. The terminal rami and tip of the antenna were removed so that the end of the antenna could be placed into the saline-filled recording electrode. The antenna was positioned on the amplifier mount at the end of the delivery tube such that the plane of the antennal branches was perpendicular to the air flow from the stimulus delivery tube. A single antennal preparation could be used for several hours with periodic additions of saline. Antennal responses were quantified by measuring the heights of the electroantennogram peaks to the nearest 0.5 mm.

Coupled Gas Chromatography–Mass Spectrometry. Electron impact (EI) mass spectra (70 eV) were taken with an H-P 6890 gas chromatograph in splitless mode interfaced to a 5973 mass selective detector. A 30-m × 0.25-mm-ID HP-5MS column was used, programmed at 50°C for 1 min, rising 10°/min to 250°C, and held for 20 min, with injector temperature 250°C, and transfer line 280°C. Compounds in the insect extract were identified by comparison of retention times and mass spectra with those of authentic standards (see below).

Formation of Adducts with 4-Methyl-1,2,4-triazoline-3,5-dione (MTAD). To determine the positions of conjugated dienes in the pheromone components, the dienes were reacted with the powerful dienophile MTAD to form Diels-Alder cycloadducts, the mass spectra of which show prominent fragment ions from cleavage of the alkyl chains from the two outer carbons of the former diene system (Young and Vouros, 1990; McElfresh and Millar, 1999a–c). A pheromone gland extract was evaporated to dryness and then treated with 5 μl of a solution

of MTAD (Aldrich, Milwaukee, Wisconsin) in methylene chloride (2 mg/ml) at room temperature. The resulting solution was analyzed immediately by GC-MS, with injector and transfer line temperatures of 300°C, and a temperature program of 100°C at 0 min, rising 15°C/min to 300°C, and held for 20 min.

Synthesis of Pheromone Components. The synthesis and purification of compounds used in field trials and as standards have been described previously (McElfresh and Millar, 1999a). GC standards of all four 10,12–16 : Ac isomers were also obtained from Darwin Reed, Plant Biotechnology Institute, National Research Council of Canada, Saskatoon, Saskatchewan, Canada.

Field Trials with Live Females. Field trials comparing the relative attractiveness of calling females of the two subspecies, Hee and Hem, to males of Hem and *H. burnsi* were conducted at the Ord Mountain site on September 28, 1996. Reared females (1–8 days old) were used as lures (mean age for Hee: 5.1 days, SD 2.75; mean age for Hem: 4.9 days, SD 2.03). Block 1 consisted of three females of each subspecies from only Aguanga Hee and Ord Mt. Hem populations. Blocks 2–4 consisted of two females each of Hee and Hem from multiple populations. Cylindrical traps were constructed from hardware cloth as previously described (McElfresh and Millar, 1999a,b), except that a 5-cm mesh chicken wire cylinder (40 cm high × 30 cm diam.) was placed around the trap with a 30-cm^2 piece of cardboard weighted with a rock placed on top. This excluded birds and provided shade for the calling female moths. Screen cages (6 cm diam. × 8 cm high, made of galvanized window screen with a 50-mm plastic Petri plate lid for the bottom) containing females were placed on the wire hooks over the funnel entrance to each trap. Traps for a given block were placed 10 m apart in straight lines. Four blocks of traps separated by a minimum of 300 m were used. Males were removed from traps at intervals throughout the day and held in bags until the completion of the trial. Whether or not the females were calling and the number of males trapped were recorded at each time interval. Counts from noncalling females were not included in the analysis. Results were analyzed by two-way ANOVA (SAS Institute, 1996).

Field Trials with Synthetic Pheromone Components. Field trials primarily were conducted at two locations: 6 km east of Aguanga, Riverside County, California, for Hee and at the base of the Ord Mountains, east of Hesperia, San Bernardino County, California for Hem. A further field trial specifically targeting *H. burnsi* was conducted approximately 27 km west of the Ord Mountains site because of the relatively low populations of this moth at the Ord Mountains site at the time of the trial. A single trial consisted of three to six blocks of replicates located at different points within the habitat. Traps were spaced approximately 10 m apart and blocks were 200 m or more apart. Two to four trap counts were made at one-day intervals, with the traps being cleaned out and rerandomized after each reading. Trap catches were tabulated either early in the morning or late in the day after moth flight had ceased.

The traps used were of the same design as those used in the live female study (above), except that the chicken wire protective cylinder was not used. Lures consisted of Wheaton 11-mm red rubber septa impregnated with heptane solutions of synthetic pheromone components + 2 drops of butylated hydroxy-toluene in heptane (10 mg/ml) as an antioxidant. Lures were suspended from wires above the funnel with bronze fish hooks.

Statistics. Comparisons of the relative amounts of compounds found in female extracts of the two subspecies were made by first converting the amount of each compound to a percentage of the amount of *E*10,*Z*12–16 : Ac, the major component of the pheromone blend of both subspecies. The proportions of each compound were then compared by a t test on the $\log_{10}$-transformed data.

A similar approach was used for comparing GC-EAD responses from male antennae stimulated with a 1 : 1 : 1 ng/μl blend of the three primary components, *E*10,*Z*12–16 : Ac, *E*10,*Z*12–16 : OH, and *E*10,*Z*12–16 : Ald. The antennal response (peak height) to each compound was converted to a proportion of the combined responses to all three, then square root arcsin transformed prior to conducting a t-test.

For the statistical analysis of field trials, day effect was eliminated by averaging the daily trap counts for each treatment in a given block. This mean was then transformed ($\sqrt{x + 0.5}$) prior to conducting a two-way ANOVA, followed by linear and quadratic contrasts. If significant effects were detected, a Student-Neuman-Keuls (SNK) test was used to separate means (SAS Institute, 1996).

RESULTS

Analysis of Female Extracts. Coupled GC-EAD analyses of female pheromone gland extracts showed that the response profiles of male antennae from both *H. electra* subspecies were similar, indicating that the female pheromone components and male antennal receptors were common to both subspecies (Figure 1). Male antennae were highly sensitive to three principal compounds, with weaker responses elicited by several other components in the extracts (Figure 1, Table 1).

Compounds in female extracts were identified by a combination of retention time matches on DB-5 (nonpolar) and DB-Wax (polar) GC columns, and mass spectral matches with synthesized standards. Reaction of conjugated dienes in the extracts with 4-methyl-1,2,4-triazoline-3,5-dione (MTAD) produced Diels-Alder adducts whose mass spectra contained ions that unequivocally located the diene positions in the original compounds (Young and Vouros, 1990; McElfresh and Millar, 1999a–c). Furthermore, *E*10,*Z*12–16 : OH, Ald, and Ac are known pheromone components of other Hemileucine moth species (McElfresh and Millar, 1999a–c), which aided in the identification of the same components in the *H. electra* subspecies. The data are summarized in Table 2.

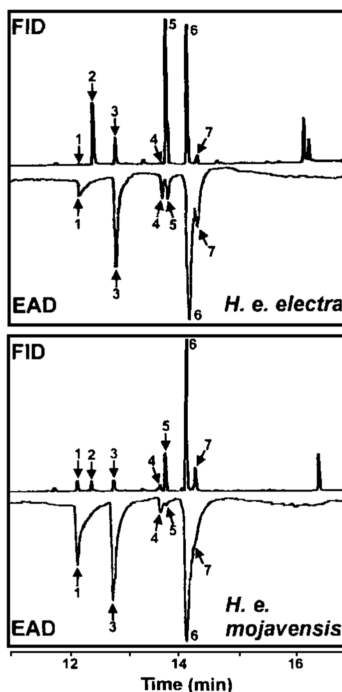

FIG. 1. Coupled gas chromatogram–electroantennograms of male *Hemileuca electra electra* and *H. e. mojavensis* antennae stimulated by single female extracts. Column: DB-5 (30 m × 0.32 mm ID, 0.25 μm film; program, 100°C for 1 min, 10°C/min to 275°C hold for 45 min). Identifications of peaks: (1) *E*10,*Z*12–16 : Ald, (2) 16 : OH, (3) *E*10,*Z*12–16 : OH, (4) *E*- and *Z*11–16 : Ac, (5) 16 : Ac, (6) *E*10,*Z*12–16 : Ac, and (7) *E*10,*E*12–16 : Ac.

The compound eliciting the largest EAD response from male antennae gave a distinct molecular ion at m/z 280 (24% of base peak at m/z 67), consistent with a 16-carbon diunsaturated acetate. The comparatively strong molecular ion peak and retention times significantly longer than 16 : Ac suggested that the double bonds were conjugated. This was confirmed by formation of the MTAD adduct, the mass spectrum of which showed a molecular ion at m/z 393 (7%), and diagnostic ions at m/z 350 (22%) and 208 (100%) from loss of the alkyl chains at positions 3 and 6 of the six-membered ring of the adduct. The diene was determined to have the *E*10,*Z*12 geometry by comparison of its retention times on DB-5 and DB-Wax GC columns with those of standards of all four stereoisomers. Only the *E*10,*Z*12 isomer matched the insect-produced peak on both columns. In total, these data unequivocally identified the insect-produced compound as *E*10,*Z*12–16 : Ac.

TABLE 1. ELECTROANTENNOGRAPHIC RESPONSES (EXPRESSED AS PERCENT OF RESPONSE TO *E*10,*Z*12–16:Ac) AND PERCENT OF MALE *Hemileuca electra electra* AND *H. e. mojavensis* ANTENNAE THAT PRODUCED A DETECTABLE RESPONSE TO COMPOUNDS IN THEIR RESPECTIVE FEMALE EXTRACTS

	% Response relative to *E*10,*Z*12–16:Ac							
	H. electra electra[a]				*H. electra mojavensis*[b]			
Compound	Mean	SD	Range	% det.[c]	Mean	SD	Range	% det.[c]
*E*10,*Z*12–16:Ac	100			100	100			100
*E*10,*Z*12–16:OH	40.2	12.2	22–62	100	56.3	26.1	11–118	100
*E*10,*Z*12–16:Ald	9.3	5.5	4–25	73	27.0	8.4	13–42	100
*E*10,*E*12–16:Ac	45.4	11.7	29–65	80	37.2	6.2	27–46	50
E/*Z*11–16:Ac	11.2	4.1	3–15	93	12.0	5.8	4–25	88
16:Ac	11.6	5.2	4–23	80	9.7	5.6	4–24	81

[a]Total of 3 males and 15 female extracts tested.
[b]Total of 4 males and 16 female extracts tested.
[c]Percent of male responses.

In similar fashion, a second compound eliciting large antennal responses from males was identified as *E*10,*Z*12–16 : OH. The compound gave a molecular ion at *m/z* 238 (16% of base peak at *m/z* 67), consistent with a diene alcohol, and the relatively large molecular ion peak and a retention time significantly longer than 16 : OH suggested a conjugated dienol. The retention time and mass spectrum matched that of an *E*10,*Z*12–16 : OH standard. The mass spectrum of the MTAD derivative gave a molecular ion at *m/z* 351 (15%), and diagnostic fragments at *m/z* 308 (31%) and 208 (100%), locating the diene in the 10,12 position. Of the four possible geometric isomers, only *E*10,*Z*12–16 : OH had identical retention times to the insect-produced compound on both GC columns.

The third, minor component that elicited strong EAD signals from male moth antennae was identified as *E*10,*Z*12–16 : Ald. A full-scan mass spectral match and retention time match was obtained from a pheromone gland extract from Hem females. There was a significant molecular ion at *m/z* 236 (16% of base peak at *m/z* 67), consistent with a conjugated 16-carbon dienal, and the mass spectrum and retention time matched those of a standard. The relatively weak molecular ion of the MTAD derivative was not seen due to the small amount of the compound in the extract, but the diagnostic fragments for location of the diene system [*m/z* 306 (40%) and 208 (100%)] were present. As with the acetate and alcohol components, the aldehyde was determined to be the *E*10,*Z*12 geometric isomer by comparison of retention times of the insect produced compound with standards. This compound was not detected in the Hee extracts by full-scan mass spectrometry due to the trace amount present, but the diagnostic MTAD derivative fragments (*m/z* 306 and 208) were found at the

TABLE 2. COMPOUNDS, METHODS OF IDENTIFICATION, AND AMOUNTS RELATIVE TO *E*10,*Z*12–16:Ac IN SINGLE FEMALE EXTRACTS OF *Hemileuca electra electra* FROM 4 MILES EAST OF AGUANGA, RIVERSIDE COUNTY, AND *H. e. mojavensis* FROM NEAR ORD MOUNTAINS EAST OF HESPERIA, SAN BERNARDINO COUNTY, CALIFORNIA

	Identification methods used[a]		Amount in single female extracts relative to *E*10,*Z*12–16:Ac										
			H. electra electra[b]				*H. electra mojavensis*[c]				*t* test		
Compound	GC	GC-MS	Mean	SD	Range	% det.[d]	Mean	SD	Range	% det.[d]	*t*	*df*	*P*
*E*10,*Z*12–16:Ac	X	X	100			100	100			100			
*E*10,*Z*12–16:OH	X	X	21.9	15.7	6.3–82	100	35.5	70	4.6–292	88	-0.419	38	0.6772
*E*10,*Z*12–16:Ald	X	X	0.45	0.26	0.1–1.1	44	3.5	2.1	0.97–8.7	76	7.23	22	<0.0001
16:Ac	X	X	203	255	23–1290	100	104	120	11–487	100	-2.36	40	0.0235
*E*10,*E*12–16:Ac	X	X	6.7	2.0	4.1–12.4	88	10.1	7.7	3.8–34	88	1.65	35	0.1087
*E*10,*E*12–16:OH	X	X[e]	2.5	2.5	0.3–9.2	64	14.6	34	0.50–119	65	1.17	24	0.2527
E/*Z*11–16:Ac	X	X[e]	2.2	2.1	0.6–10.9	88	4.0	2.9	0.70–10.6	59	2.36	32	0.0243
*Z*10–16:Ac	X		0.43	0.14	0.2–0.6	24	1.0	0.64	0.24–2.2	41	2.09	11	0.0605
16:OH	X	X	92	95	3.8–364	100	111	197	2.6–691	100	-1.53	40	0.1349

[a]GC = retention time matches on two columns (see Methods and Materials) with authentic standards. GC-MS = mass spectral match with authentic standards.
[b]Means of 25 female extracts.
[c]Means of 17 female extracts.
[d]Percent of female extracts with detectable amounts.
[e]GC-MS on composite extracts.

correct retention time, providing strong evidence that the aldehyde was indeed present. Furthermore, retention time matches were obtained on both GC columns (Table 2).

Several other compounds were also identified in the extracts by comparison of mass spectra and retention times on both polar and nonpolar GC columns with those of standards. These other compounds included small amounts of the *E*10,*E*12 isomers of both the diene acetate and diene alcohol (Table 2), 16 : OH, and 16 : Ac. 16 : OH elicited no detectable EAD signal from male antennae, whereas 16 : Ac elicited a small response.

Trace amounts of several monoene acetates also were found in extracts, including *Z*10–16 : Ac, and *E*- and *Z*11–16 : Ac. These compounds were tentatively identified by careful comparison of peak intensities and retention times on DB-Wax and DB-5 columns, as described in detail previously (McElfresh and Millar, 1999a,b). Corroborating evidence for the identification of *Z*- and *E*11–16 : Ac was provided by comparison of mass spectra obtained from analysis of composite extracts of females of both subspecies with those of standards.

Because *E*10,*Z*12–16 : Ac elicited the largest antennal responses from males of both Hee and Hem and appeared to be the major component of the pheromone blend for each subspecies, for comparative purposes the amounts of the other compounds in the extracts were expressed as a proportion of *E*10,*Z*12–16 : Ac in a given extract (Table 2). Hem gland extracts had approximately eight times as much *E*10,*Z*12–16 : Ald (relative to *E*10,*Z*12–16 : Ac) as Hee extracts, whereas Hee extracts contained about twice as much 16 : Ac as Hem extracts (Table 2). The actual proportion of *E*10,*Z*12–16 : Ald in the Hee population may in reality be much lower, because only 44% (11 of 25) of the female extracts tested possessed measurable amounts, whereas 76% of the Hem extracts had measureable amounts of *E*10,*Z*12–16 : Ald. The relative amounts of the other major EAD-active component, *E*10,*Z*12–16 : OH, were not significantly different between subspecies. The only other significant difference detected between the female extracts was in the combined amount of *E*- and *Z*11–16 : Ac, with Hem possessing nearly twice as much as Hee. Substantial intraspecific variation in the relative amounts of the other identified compounds in the extracts confounded efforts to detect interspecific differences in their amounts.

GC-EAD Results. Differences in antennal response profiles between males of the two subspecies could not be determined reliably from comparison of responses to female extracts because of differences in the relative and absolute amounts of compounds present (Table 2). However, significant differences between subspecies were found when antennae were challenged with a 1 : 1 : 1 blend of *E*10,*Z*12–16 : Ac, *E*10,*Z*12–16 : OH, and *E*10,*Z*12–16 : Ald standards in GC-EAD trials (Figure 2, Table 3). Following conversion of responses to individual compounds to proportions of the summed responses to the three test compounds, Hem antennae produced significantly smaller responses to

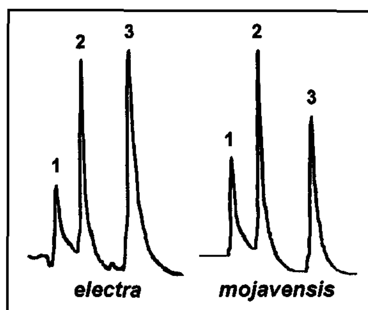

FIG. 2. Coupled gas chromatogram–electroantennograms of male *Hemileuca electra electra* and *H. e. mojavensis* antennae stimulated by a 1 : 1 : 1 ng/μl mixture of *E*10,*Z*12–16 : Ald (peak 1), *E*10,*Z*12–16 : OH (peak 2), and *E*10,*Z*12–16 : Ac (peak 3). Column and conditions were as in Figure 1.

*E*10,*Z*12–16 : Ac and significantly larger responses to *E*10,*Z*12–16 : Ald than Hee antennae. These data suggest that the relative numbers of receptors for the two compounds may differ between males of the two subspecies. Responses to *E*10,*Z*12–16 : OH were not significantly different between the two subspecies.

Field Trials with Live Females. A field trial (September 28, 1996; Ord Mountains site) with calling females of the two subspecies demonstrated significant differences in their attractiveness to male moths. Hem males showed a 5 : 1 preference for females of their own subspecies (two-way ANOVA for female subspecies effect; $F = 34.1$, $df = 1, 3$, $P = 0.01$, total males trapped, 2626), despite the potentially confounding effects of the large variation in the number of males attracted by individual females (range of Hem males attracted to individual Hee females = 3–226; to individual Hem females, 7–433). In addition, 40 *H. burnsi* males were attracted to Hee females, whereas only a single *H. burnsi* male was

TABLE 3. ELECTROANTENNOGRAPHIC RESPONSES OF MALE *Hemileuca electra electra* AND *H. e. mojavensis* ANTENNAE TO 1 : 1 : 1 MIXES OF SYNTHETIC STANDARDS[a]

Compound	*H. e. electra* (mean ± SD)	*H. e. mojavensis* (mean ± SD)	*t* test: *t*	*t* test: *df*	*t* test: *P*
*E*10,*Z*12–16:Ac	43.1 ± 8.8	28.5 ± 8.4	4.77	32	<0.0001
*E*10,*Z*12–16:OH	42.6 ± 10.1	48.0 ± 9.4	−1.52	32	0.1388
*E*10,*Z*12–16:Ald	14.2 ± 4.0	23.5 ± 3.0	−7.43	32	<0.0001

[a]Responses are expressed as a percentage of the combined responses to *E*10,*Z*12–16:Ac, *E*10,*Z*12–16:OH, and *E*10,*Z*12–16:Ald. $N = 17$ for both populations.

attracted to an Hem female (two-way ANOVA for female effect: $F = 41.2$, $df = 1, 2$, $P = 0.024$). Some degree of allochrony also was observed, with the majority of Hem males (88% of 1062 trapped in one block) being trapped before 1200 hr, whereas the majority of *H. burnsi* males were attracted between 1500 hr and dusk (14 of 17 males trapped in one block). Finally, it was observed that if mating was prevented, females of both subspecies continued to call throughout the day.

Field Trials with Synthetic Pheromone Lures. Five field experiments were conducted to assess responses of males of each subspecies to different pheromone blends. In field trials testing different doses of *E*10,*Z*12–16 : OH in combination with a fixed blend of *E*10,*Z*12–16 : Ac, *E*10,*Z*12–16 : Ald, and 16 : Ac, responses of males of the two subspecies overlapped considerably. There were no significant differences in the numbers of Hem males attracted to lures containing 1–100 μg of *E*10,*Z*12–16 : OH, whereas Hee males were most attracted to lures with 10 or 33 μg *E*10,*Z*12–16 : OH (Figure 3). The two subspecies also responded similarly to variable doses of *E*10,*Z*12–16 : Ac, with attraction of males of both subspecies being proportional to the dose of *E*10,*Z*12–16 : Ac (Figure 4).

The two subspecies varied in their behavioral responses to 16 : Ac. There were no significant differences in the numbers of Hem males attracted to lures containing 0–1000 μg 16 : Ac, whereas Hee males were optimally attracted to lures with from 33 to 1000 μg/septum (Figure 5). These data suggest that 16 : Ac has some role in the pheromone blend of Hee, but it is of no importance to Hem.

There were distinct differences in the responses of Hem and Hee males to different doses of *E*10,*Z*12–16 : Ald in two sequential experiments. The first experiment used blends of *E*10,*Z*12–16 : Ac + *E*10,*Z*12–16 : OH (100 : 10 μg) admixed with variable doses of *E*10,*Z*12–16 : Ald. The response profiles of males of the two subspecies were clearly different, with Hee males responding optimally to 0.3- and 1-μg doses of *E*10–*Z*12–16 : Ald, with decreased responses to higher doses. In contrast, Hem males were minimally attracted to blends containing lower doses of *E*10,*Z*12–16 : Ald, and maximally attracted to blends containing 10–100 μg *E*10,*Z*12–16 : Ald (Figure 6).

These results were corroborated in a second field trial with more complex blends approximating the entire suite of compounds obtained from female *H. electra* and *H. burnsi* (Figure 7). In this trial, both Hee and *H. burnsi* (which are allopatric) were attracted optimally by lures containing 1 μg *E*10,*Z*12–16 : Ald, with significantly lower trap catches to higher doses of *E*10,*Z*12–16 : Ald (Figure 7), whereas Hem males were optimally attracted to lures containing doses of 10 or 100 μg *E*10,*Z*12–16 : Ald. These results, coupled with the results from female pheromone gland analyses and male antennal response profiles suggest that the relative amount of *E*10,*Z*12–16 : Ald in a pheromone blend is an important factor in the reproductive isolation of the sympatric Hem and *H. burnsi*.

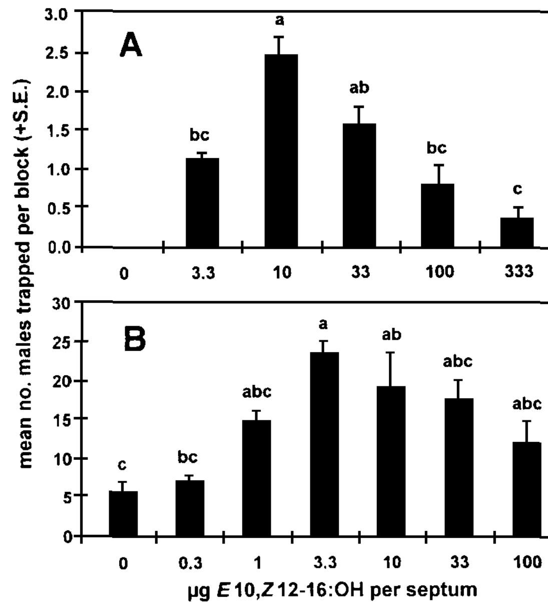

FIG. 3. (A) Trap catches of *Hemileuca electra electra* near Aguanga, Riverside Company, California, November 7–9, 1993, with lures consisting of *E*10,*Z*12–16 : Ac, *E*10,*Z*12–16 : Ald, and 16 : Ac (100 : 1 : 33 μg/septum, respectively) with variable doses of *E*10,*Z*12–16 : OH. Total number of moths trapped = 56. Two-way ANOVA for blend effect $F = 9.1$, $df = 4, 8$, $P = 0.0046$; linear contrast $F = 17.0$, $df = 1, 8$, $P = 0.0034$, quadratic contrast $F = 12.3$, $df = 1, 8$, $P = 0.0080$. Treatments with different letters are significantly different (SNK, $\alpha = 0.05$). (B) Trap catches of *H. e. mojavensis* near Ord Mountains, San Bernardino County, California, September, 26–28, 1994, with lures consisting of *E*10,*Z*12–16 : Ac and *E*10,*Z*12–16 : Ald (100 : 20 μg/septum, respectively) and variable rates of *E*10,*Z*12–16 : OH. Total moths trapped = 597. Two-way ANOVA for blend effect $F = 5.2$, $df = 6, 12$, $P = 0.0075$; linear contrast $F = 9.3$, $df = 6, 12$, $P = 0.0100$, quadratic contrast $F = 17.4$, $df = 1, 12$, $P = 0.0013$. Treatments with different letters are significantly different (SNK, $\alpha = 0.05$).

Several other compounds found in female extracts were tested for activity in field trials for both Hem and Hee. A single rate of *E*10,*E*12–16 : Ac (10 μg/septum) added to a standard blend consisting of 100 : 10 : 1 μg/septum of *E*10,*Z*12–16 : Ac to *E*10,*Z*12–16 : OH to *E*10,*Z*12–16 : Ald did not increase trap catches over that of the standard blend for Hee (two-way ANOVA for additive effect; $F = 2.24$, $df = 1, 2$, $P = 0.28$), nor did the addition of doses of 0–100 μg/septum of *E*10,*E*12–16 : Ac added to a standard blend of 100 : 10 : 33 *E*10,*Z*12–16 : Ac to *E*10,*Z*12–16 : OH to *E*10,*Z*12–16 : Ald have any effect on

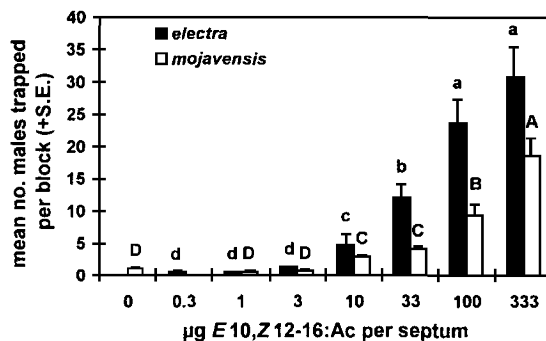

FIG. 4. Attraction of *Hemileuca electra* to lures containing variable doses of *E*10,*Z*12–16 : Ac. Field sites were as described in Figure 3. *H. e. electra* lures contained *E*10,*Z*12–16 : OH and *E*10,*Z*12–16 : Ald (10 : 1 μg/septum, respectively) + variable doses of *E*10,*Z*12–16 : Ac. Trial date: October 9–11, 1994. Total moths trapped = 417. Treatments with different letters are significantly different (SNK, $\alpha = 0.05$). Two-way ANOVA for *E*10,*Z*12–16 : Ac rate effect, $F = 55.2$, $df = 6, 12$, $P = 0.0001$; linear contrast $F = 307.6$, $df = 1, 12$, $P = 0.0001$, quadratic contrast $F = 16.7$, $df = 1, 12$, $P = 0.0015$. *H. e. mojavensis* lures contained *E*10,*Z*12–16 : OH and *E*10,*Z*12–16 : Ald (10 : 33 μg/septum, respectively) + variable doses of *E*10,*Z*12–16 : Ac. Trial date: September 30-October 3, 1994. Total moths trapped = 333. Treatments with different letters are significantly different (SNK, $\alpha = 0.05$). Two-way ANOVA for *E*10,*Z*12–16 : Ac rate effect, $F = 78.7$, $df = 6, 12$, $P = 0.0001$; linear contrast $F = 390.3$, $df = 1, 12$, $P = 0.0001$, quadratic contrast $F = 77.4$, $df = 1, 12$, $P = 0.0001$.

trap captures of male Hem (two-way ANOVA for *E*10,*E*12–16 : Ac effect; $F = 3.20$, $df = 4, 8$, $P = 0.0757$). Addition of monounsaturated acetates in proportions found in extracts (*Z*10–16 : Ac, 0.45 μg/septum; *E*11–16 : Ac, 0.75 μg/septum; *Z*11–16 : Ac, 0.5 μg/septum) to the Hee blend or to the Hem blend (*Z*10–16 : Ac, 1 μg/septum; *E*11–16 : Ac, 1 μg/septum; *Z*11–16 : Ac, 2 μg/septum) did not result in increased trap catches for either population (two-way ANOVA, $F = 4.39$, $df = 4, 8$, $P = 0.036$; SNK, $\alpha = 0.05$, not significant for Hee, with the standard treatment without any of the monounsaturated acetates attracting the most moths, $N = 2439$; for Hem, two-way ANOVA, $F = 1.32$, $df = 4, 8$, $P = 0.341$, $N = 4799$).

DISCUSSION

The formation of the cycloadducts of the conjugated diene components of the pheromone with MTAD proved to be exceptionally useful for two reasons. First, the cycloadducts unequivocally located the position of the diene, regardless

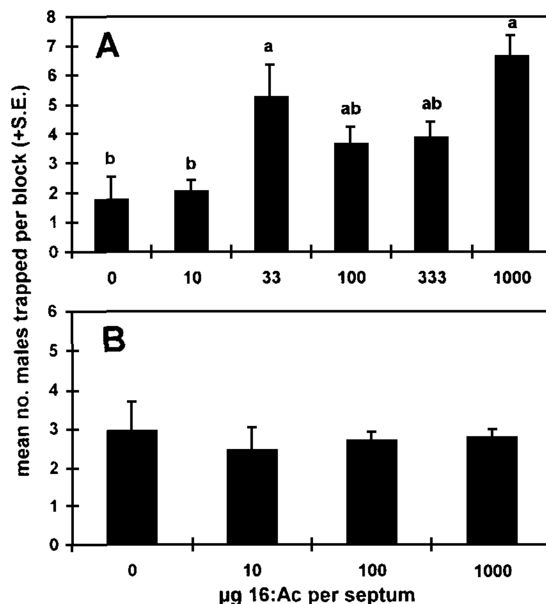

FIG. 5. *Hemileuca electra* pheromone trials for optimization of 16 : Ac doses. Field sites were as in Figure 3. (A) *H. e. electra* field trial October 26–29, 1993. Lures: *E*10,*Z*12–16 : Ac, *E*10,*Z*12–16 : OH, and *E*10,*Z*12–16 : Ald (100 : 25 : 1 μg/septum, respectively) + variable doses of 16 : Ac. Total moths trapped = 406. Treatments with different letters are significantly different (SNK, α = 0.05). Two-way ANOVA for 16 : Ac rate effect, F = 6.22, df = 5, 25, P = 0.0007; linear contrast F = 22.5, df = 1, 25, P = 0.0001, quadratic contrast F = 0.26, df = 1, 25, P = 0.6143. (B) *H. e. mojavensis* field trial October 3–8, 1994. Lures: *E*10,*Z*12–16 : Ac, *E*10,*Z*12–16 : OH and *E*10,*Z*12–16 : Ald (100 : 10 : 33 μg/septum, respectively) + variable rates of 16 : Ac. Total moths trapped = 123. Treatments with different letters are significantly different (SNK, α = 0.05). Two-way ANOVA for 16 : Ac rate effect, F = 0.1, df = 3, 6, P = 0.9622; linear contrast F = 0.0, df = 1, 6, P = 0.9430, quadratic contrast F = 0.2, df = 1, 6, P = 0.7110.

of the functional group of the pheromone component. Second, because cleavage of the alkyl chains at the 3 and 6 positions of the ring is highly favored, most of the fragmentation is confined to these two fragments, which give very strong signals. Consequently, it appears possible to detect smaller quantities of the derivative than of the original underivatized compound, in which extensive fragmentation occurs. For example, in the analyses reported above, *E*10,*Z*12–16 : Ald was not detected in the underivatized Hee extract by full-scan GC-MS, whereas the diagnostic fragment ions of the derivative were clearly visible.

Pheromone systems based on *E*10,*Z*12–16 carbon diene components are

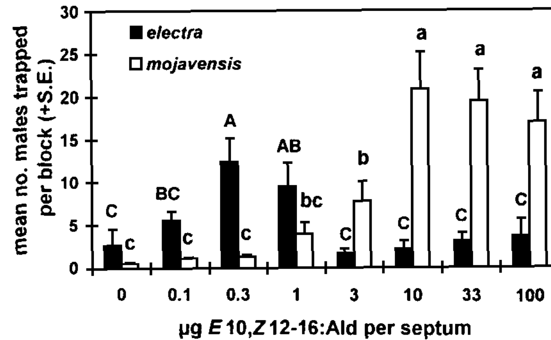

FIG. 6. *Hemileuca electra* pheromone trials for optimization of *E*10,*Z*12–16 : Ald doses. Lures: *E*10,*Z*12–16 : Ac and *E*10,*Z*12–16 : OH (100 : 10 μg/septum, respectively) + variable doses of *E*10,*Z*12–16 : Ald. Field sites were as in Figure 3. *H. e. electra* field trial, October 11–14, 1993. Total moths trapped = 351. Treatments with different letters are significantly different (SNK, $\alpha = 0.05$). Two-way ANOVA for *E*10,*Z*12–16 : Ald rate effect, $F = 8.4$, $df = 7, 14$, $P = 0.0004$; linear contrast $F = 3.9$, $df = 1, 14$, $P = 0.0687$, quadratic contrast $F = 8.4$, $df = 1, 14$, $P = 0.0115$. *H. e. mojavensis* field trial, September 27–30, 1994. Total moths trapped = 606. Treatments with different letters are significantly different (SNK, $\alpha = 0.05$). Two-way ANOVA for *E*10,*Z*12–16 : Ald rate effect, $F = 26.9$, $df = 7, 14$, $P = 0.0001$; linear contrast $F = 165.7$, $df = 1, 14$, $P = 0.0001$, quadratic contrast $F = 0.03$, $df = 1, 14$, $P = 0.8731$.

emerging as a shared motif among saturniid moths in the genus *Hemileuca*. We have now identified *E*10,*Z*12–16 : Ac, *E*10,*Z*12–16 : OH, and *E*10,*Z*12–16 : Ald as pheromone components of at least five species, including *H. eglanterina* (McElfresh and Millar, 1999a), *H. nuttalli* (McElfresh and Millar, 1999b), *H. maia* (McElfresh et al., unpublished data), and the two species in this study, *H. electra* and *H. burnsi*. These compounds have also been found in several other *Hemileuca* species still under investigation (McElfresh and Millar, unpublished data). Furthermore, *E*10,*Z*12–16 : Ac and *E*10,*Z*12–16 : OH are also pheromone components for the related saturniid species *Coloradia velda* (McElfresh and Millar, 1999c). Prior to our studies, only a single species, *Rondotia menciana* Moore, had been recorded to utilize *E*10,*Z*12–16 : Ac (Dai et al., 1987), although the corresponding alcohol and aldehyde are known or suspected pheromone components for several bombycid and sphingid species (Arn et al., 1995).

It is also of interest that the sex pheromone of Hee is very similar to that of *H. eglanterina* from the San Gabriel Mountains (McElfresh and Millar, 1999a). Attraction of male *H. eglanterina* to female *H. electra* artificially moved to *H. eglanterina* habitat has been observed (Tuskes et al., 1996), but the two species are entirely allopatric, and have quite different ecologies. Consequently, in the absence of any selective force inducing divergence in blends, the pheromone

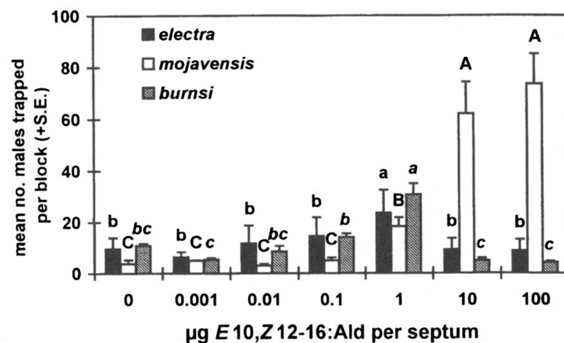

FIG. 7. Trap catches of *Hemileuca electra* and *Hemileuca burnsi* in field trials for optimization of the *E*10,*Z*12–16 : Ald dose. Field sites for *H. e. electra* and *H. e. mojavensis* were as described in Figure 3. Field site for *H. burnsi*: 27 km west of Ord Mountains site, San Bernardino County, California. Lures: *E*10,*Z*12–16 : Ac, *E*10,*Z*12–16 : OH, *E*10,*Z*12–16 : Ac, 16 : Ac, and 16 : OH (100 : 10 : 100 : 1000 : 60 μg/septum, respectively) + variable rates of *E*10,*Z*12–16 : Ald. Treatments with different letters are significantly different (SNK, $\alpha = 0.05$). *H. e. electra* field trial, October 11–13, 1995. Total of 752 moths trapped. Two-way ANOVA for *E*10,*Z*12–16 : Ald rate effect, $F = 6.6$, $df = 6, 12$, $P = 0.0029$; linear contrast $F = 1.4$, $df = 1, 12$, $P = 0.2579$, quadratic contrast $F = 12.1$, $df = 1, 12$, $P = 0.0045$. *H. e. mojavensis* field trials October 7–8 and 14–15, 1995. Total of 1762 moths trapped. Two-way ANOVA for *E*10,*Z*12–16 : Ald rate effect, $F = 84.9$, $df = 6, 12$, $P = 0.0001$; linear contrast $F = 402.5$, $df = 1, 12$, $P = 0.0001$, quadratic contrast $F = 76.7$, $df = 1, 12$, $P = 0.0001$. *H. burnsi* field trial October 7–8, and 14–15, 1995. Total of 946 moths trapped. Two-way ANOVA for *E*10,*Z*12–16 : Ald rate effect, $F = 17.2$, $df = 6, 12$, $P = 0.0001$; linear contrast $F = 0.0$, $df = 1, 12$, $P = 0.8902$, quadratic contrast $F = 22.6$, $df = 1, 12$, $P = 0.0005$.

signaling systems of the two species appear to have remained remarkably similar.

When originally coined, the term "character displacement" was defined as the phenomenon of greater divergence in traits between two closely related species in areas of sympatry than between allopatric populations (Brown and Wilson, 1956). "Character displacement" and the related term "reinforcement," were further refined by Butlin (1987), with "reinforcement" being reserved for the divergence of traits during speciation, when some degree of hybridization is still possible between the diverging populations, and "character displacement" reserved for the divergence of traits that are coincidentally shared by sympatric species after speciation has occurred, so that hybridization is impossible. Under Butlin's (1987) definition, reinforcement may form part of the speciation process, whereas character displacement provides a means for reducing interference and increasing the reliability of signals used by sympatric species.

It can be reasonably argued, but not proven, under either Brown and Wilson's (1956) original definition, or under Butlin's (1987) later refinement, that the differences in the pheromone signaling systems of Hem and Hee may constitute a case of reproductive character displacement, in the context of a subset of Hem populations evolving to minimize interference from sympatric *H. burnsi*, with which they cannot hybridize. We have presented evidence for differences between Hem and Hee populations in three distinct parts of the pheromone signaling process. First, Hem females produce a significantly different pheromone blend than Hee females, but there is still cross attraction between the two subspecies. Second, antennae of male Hem are tuned differently than those of Hee males, being significantly more responsive to the increased quantity of *E*10,*Z*12–16 : Ald produced by Hem females. Third, although the integration of the component parts of the pheromone signal in the brain can be inferred only indirectly through male behavioral responses, it is clear that Hem males respond optimally to a different pheromone blend than Hee males. Thus, it is clear that the two subspecies differ in their pheromone systems, and it is reasonable, but cannot be proven, that interference from the congeneric *H. burnsi* provided a selective force to drive this change.

The particular mechanism that is used to limit cross-attraction between Hem and *H. burnsi* in their regions of sympatry, that is, subtle alterations in the ratio of pheromone components by female Hem, and corresponding alteration in both the male antennal and behavioral responses, also supports a case for reproductive character displacement. All three alterations require only minor modification of preexisting traits, rather than the evolution of entirely new ones (Roelofs and Brown, 1982). This is important, because the demonstrated lack of genetic linkage between the male and female components of pheromone signaling systems of moths (Roelofs et al., 1987) would retard the development of novel pheromone components, whereas interindividual variation in pheromone production, reception, and response, as demonstrated by the data in this report and as demonstrated in a number of other lepidopteran species (reviewed in Linn and Roelofs, 1995), provide a scenario under which selection pressure could drive modification of existing pheromone systems.

Several cases of variation in lepidopteran mating systems have been suggested as possible examples of reproductive character displacement, and a number of examples have been suggested from other insect orders (e.g., Odonata, Diptera, and Orthoptera) (Howard, 1993). Among the few cases reported, reproductive character displacement was invoked by Löfstedt (1991) to explain the pattern of variation in mating systems within a group of closely related species of ermine moths (Yponomeutidae). Another frequently cited example involves *Archips argyrospilus* (Walker) (Cardé et al., 1977; Butlin, 1995), in which male moths from New York populations responded to a more narrowly defined blend of compounds than moths from British Columbia. The narrowing of the range

of acceptable pheromone blends in the New York population was hypothesized to be the result of interference from a sibling species, *A. mortuanus* Kearfoot, present in New York but not in British Columbia. Differences in the pheromone systems of populations of the oblique-banded leafroller in eastern and western North America are also hypothesized to have arisen as a result of interference from sympatric species (Thomson et al., 1991).

Other cases of intraspecific variation in sex pheromone-mediated mating systems have been documented, both between geographically separated populations (e.g., Töth et al., 1992), and even within single populations (e.g., Klun et al., 1975). However, in most of the documented cases, the context in which intraspecific variation may have evolved has been obscured because the distributions of the particular species and populations are a reflection of human activities, such as movement and cultivation of host plants worldwide, rather than naturally evolved distributions. For example, there are two distinct pheromone races of *Ostrinia nubilalis*, the European corn borer, which are frequently found in sympatry and which produce viable and fertile offspring when mated in the laboratory (Klun et al., 1975). However, under natural conditions, mating between the two races is strongly disfavored by the behavioral barrier imposed by the lack of attraction between males and females of the two races. The pheromone signaling system of another economically important species, *Agrotis segetum*, the turnip moth, varies with geography (Töth et al., 1992), but the significance of the documented variations and the forces which caused them to arise are difficult to discern.

The system described here has a number of advantages over the previously presented examples. First, the populations involved are clearly closely related, and there is documented evidence of gene flow between the subspecies (Tuskes and McElfresh, 1995). Second, the distributions of Hee, Hem, and *H. burnsi* are the result of natural forces and are unlikely to have been altered significantly by human activities, as is often the case with species studied. Finally, the geographic distances between the populations studied here (<100 km) are significantly smaller than those of most previous examples, and there are no insurmountable geographic barriers between populations, so that contact between the two subspecies, and between Hem and *H. burnsi*, occurs during each flight period.

In summary, the sex attractant pheromone blends of two subspecies of the saturniid moth *H. electra* have been identified. Females of both subspecies produce blends of *E*10,*Z*12–16 : Ac, *E*10,*Z*12–16 : OH, and *E*10,*Z*12–16 : Ald, in slightly different ratios. Males of each subspecies were shown to be sensitive to these different ratios, being most attracted by females or synthetic blends mimicing females of their own subspecies. The differences in the pheromone systems may have arisen due to interference from *H. burnsi*, which is sympatric with only one of the two subspecies. This shift in the pheromone signaling system of Hem may constitute a case for reproductive character displacement.

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AIRBORNE CHEMICAL COMMUNICATION IN THE WOLF SPIDER *Pardosa milvina*

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Abstract—Most studies involving chemical communication in spiders focus on contact pheromones attached to spider silk. Here we tested if males of the wolf spider *Pardosa milvina* use airborne pheromones to identify, locate, and follow females. Using a two-choice olfactometer, we tested the response of adult male *P. milvina* to a number of potential chemical cues while controlling for concomitant visual and vibratory stimuli. An airborne chemical cue from adult virgin female *P. milvina* elicited a positive taxis response from the male. We also tested adult male responses to penultimate instar female *P. milvina*, one adult male *P. milvina*, and two adult males together. In each case, test males showed no attraction to the stimuli. Additional experiments were run with pitfall traps baited with adult virgin female *P. milvina* as attractants. Again, we controlled for visual and vibratory cues from females. Pitfall traps containing virgin females captured significantly more males than control traps. Collectively, these experiments demonstrate evidence of an airborne sex pheromone in *P. milvina*.

Key Words—*Pardosa milvina*, Lycosidae, airborne, sex pheromone, wolf spider, olfactometer, pitfall traps.

INTRODUCTION

Pheromone-based communication has been extensively studied among many species (Shorey, 1976; Bradbury and Vehrencamp, 1998), while research on chemical communication among spiders has lagged behind (reviewed in Tietjen and Rovner, 1982; Foelix, 1996). Most experimental studies have focused primarily on pheromones deposited on webs (Suter and Renkes, 1984; Suter and

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Hirscheimer, 1986; Schulz and Toft, 1993; Coyle and Shear, 1981), draglines (Dondale and Hegdekar, 1973), or the substratum (Richter et al., 1971; Tietjen, 1979). Typically, spiders detect sex pheromones by making physical contact with either another spider, silk from a dragline, or a web. However, there has been evidence of direct airborne chemical communication among some species of web-building spiders. Newly molted female *Nephila clavata* (Araneae, Tetragnathidae) produce a distinct chemical cue after molting. This stimulates males to induce mating when females are still vulnerable. In addition, these males then release similar mimic chemicals to attract prey (Miyashita and Hayashi, 1996). In the Sierra dome spider (*Linyphia litigiosa*) (Linyphiidae), airborne pheromones are so important to mate attraction that, upon locating a virgin female's web, the male wads up the web to prevent further pheromone dispersion (Watson, 1986).

Ground-dwelling wolf spiders (Araneae, Lycosidae) live in low vegetation and leaf litter (Hallander, 1967; Bultman and Uetz, 1982), a habitat that may restrict visual or vibratory communication (Scheffer et al., 1996). Experiments on pheromone use in lycosids has focused mainly on pheromones associated with spider silk (i.e., contact pheromones) (Hegdekar and Dondale, 1969; Richter et al., 1971; Sarinana et al., 1971; Dondale and Hegdekar, 1973). These studies show that male lycosids, including *Pardosa* spp., respond to contact pheromones, but show no clear evidence for male orientation based on airborne sex pheromone detection. Hegdekar and Dondale (1969) concluded that olfactory stimuli are not likely to be involved in the sexual behavior of male lycosids. However, Richter et al. (1971) demonstrated that olfaction does at least play a role in stimulating courtship in the wolf spider *Pardosa amentata* and that females with sealed spinnerets (therefore, no silk production) elicit a search response from the male. Furthermore, Tietjen (1979) found either orthokinetic or orientation responses in two *Schizocosa* spp. to airborne stimuli from hidden females.

In the present study, we examined the use of an airborne pheromone in the thin-legged wolf spider *Pardosa milvina* (Araneae, Lycosidae). We presumed that, if present, the pheromone would most likely be sex-related, so we specifically investigated whether males detect and respond to a chemical released by females. We presumed that conspecifics that were not mature virgin females would elicit no attraction response from males. In both laboratory and field studies, we observed the responses of males to a chemical released into the air by adult virgin females, and we contrasted these responses with those from any possible airborne stimuli from juvenile and male conspecifics.

METHODS AND MATERIALS

Study Species. Pardosa milvina are relatively small wolf spiders (females weigh approximately 20 mg) that actively search for prey as they move across

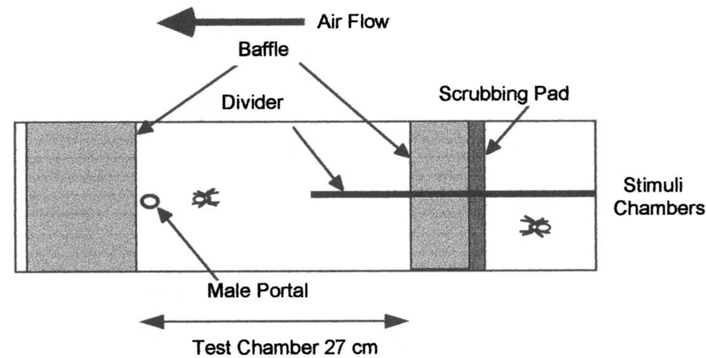

FIG. 1. Diagram (top view) of the two-choice olfactometer used to test male *Pardosa milvina* for positive taxis response.

the ground and low vegetation. *Pardosa milvina* used in these experiments were collected in soybean fields at Miami University Ecology Research Center (ERC) (Butler County, Ohio). Juvenile spiders were collected and reared in the laboratory (two to three weeks) until maturity to ensure virgin mating status of males and females. Males and immature *P. milvina* used in tests were caught three to four days prior to experimentation and were released after testing. All animals were fed three fruit flies (*Drosophila melanogaster*) 24 hr prior to their experimental trial to control for hunger. Spiders in the lab were kept in separate vials (8 cm × 3 cm diam.), provided with water, and kept on a 12L : 12D photoperiod. Adult female *P. milvina* are difficult to distinguish from other *Pardosa* species while alive. Therefore, we verified the species of all females used in trials by preserving and examining the external genitalia of each individual after completion of experiments.

Olfactometer Design. Laboratory trials were held in a two choice linear olfactometer (Figure 1). One end was connected to a fan that pulled air at 50–80 cm/min, thus creating a one-way airflow system. Screens and baffling were used on both ends of the test chamber to maintain even flow of the air and prevent contact between stimulus and test spiders.

The potential source of chemical stimulus was randomly assigned for each trial to one side of the divided tube in the upwind end. The stimulus spider was allowed to acclimate in the olfactometer for 5 min prior to introduction of the test male. We then covered the test chamber with a metal sleeve to eliminate external visual cues presented to the test male as well as to eliminate any possible visual detection of the female. An adult male was then placed into the test chamber by a portal in the downwind end. After 10 min, the sleeve was removed, and the location of the male spider noted. The tube system and screening were rinsed

before every trial with 70% ethanol to remove any silk or pheromones deposited by the spiders and then allowed to dry.

Behavioral Assay. Adult males were tested in the olfactometer with adult virgin female *P. milvina* ($N = 17$) and with immature penultimate instar female *P. milvina* ($N = 13$). In addition, males were tested with one adult male as a stimulus ($N = 15$), and with two adult males together in a stimulus chamber ($N = 15$). No test or stimulus spider was used more than once. Control trials were held with no stimulus in either chamber ($N = 10$).

Male *P. milvina* were found in three places within the olfactometer. If the male was found on the screen divider that separated him from the potential stimulus, we recorded it as a positive choice. If the male was found on the screen divider near the empty chamber, we recorded it as a negative choice. If the male was located in any other place within the olfactometer, we determined that no choice was made. We tested for nonrandom male choice using a χ^2 goodness-of-fit test.

Field Trials. The field trials were conducted in the soybean plots of the Ecology Research Center in July 1997, when *P. milvina* populations were at an observably high density and reproductive peak. Pitfall traps baited with female *P. milvina* in a screen-topped vial (10 cm × 3 cm diam.) were placed into pitfall cups (14 cm deep × 11 cm diam.). The cups were buried such that their lips were flush with the soil surface. This set-up allowed airborne chemicals produced by the females to diffuse out, but eliminated visual and vibratory communication. Traps were run with adult virgin females ($N = 17$) or empty vials (controls; $N = 8$) for a 9-hr period (08 : 00–15 : 00 hr). Traps were set at randomly determined locations separated by 20–65 m. At the end of each trial, the numbers of male *P. milvina* captured were recorded. We used an unpaired t test to compare the number of males in female-baited traps vs. the number in empty control traps.

RESULTS

Olfactometer Tests. The olfactometer results show that males chose unmated females significantly more often than an empty chamber ($\chi^2 = 8.94$, $df = 2$, $P = 0.011$) (Table 1). Of the males tested, 65% chose the female stimulus chamber over the empty chamber. Although male *P. milvina* responded to immature females in a nonrandom way ($\chi^2 = 11.2$, $df = 2$, $P = 0.003$), they showed a lack of attraction to the stimulus (Table 1). Seventy-seven percent did not make a choice. The male tested with one male ($\chi^2 = 3.6$, $df = 2$, $P = 0.165$) and two-male trials ($\chi^2 = 10.8$, $df = 2$, $P = 0.004$) showed results similar to the immature trials (Table 1). The results differed from random, but no attraction to the stimulus was shown. In the single male trials, 53% chose not to make a choice, with only 13% choosing the male chamber. The two-male trials had 73% no choice responses, while only 13% chose the chamber holding the two males.

TABLE 1. MALE RESPONSE TO POTENTIAL STIMULI IN TWO-CHOICE OLFACTOMETER

Male Response	Virgin female (N = 17)	Immature female *P. milvina* (N = 13)	1 Male (N = 15)	2 males (N = 15)
Positive choice	11	2	2	2
Negative choice	1	1	5	2
No choice	5	10	8	11
χ^2	8.94	11.2	3.6	10.8
df	2	2	2	2

Field Trials. The field trials showed that pitfall traps baited with unmated females caught significantly more male *P. milvina* than control traps ($t = -2.420$, $df = 19$, $P = 0.0257$). Female baited traps captured a mean of 1.692 ± 0.263 males, whereas control traps caught an average of 0.750 ± 0.250 males.

DISCUSSION

Male *P. milvina* clearly respond to an airborne chemical cue. Under laboratory conditions, male *P. milvina* are attracted to adult virgin female *P. milvina* via an airborne pheromone, while immature females and other adult males elicit no attraction response from the male. Males exhibit a nonrandom response to juveniles and males by showing no choice significantly more often than expected by chance. This suggests that a cue from these latter stimulus sources may inhibit movement. Pitfall traps containing unmated females caught significantly more males, which indicates that an airborne sex pheromone produced by females is an effective form of communication under field conditions.

Richter et al. (1971) found olfactory cues in *Pardosa amentata* to play only a minor role in sexual communication relative to silk-associated contact pheromones. Tietjen (1979) found that males of two *Schizocosa* spp. of wolf spider were affected by female airborne pheromones. In contrast to these and the present studies, other olfaction testing on lycosids, including *Pardosa* spp., has shown either no attraction response of males to females or ambiguous results. *Lycosa rabida* and *Lycosa punctulata* showed no response when tested for detection of possible airborne pheromones (Tietjen, 1979). In experiments conducted by Hegdekar and Dondale (1969), two *Schizocosa* spp. and two *Pardosa* spp. (excluding *P. milvina*) showed positive, although identical reactions of males to contact pheromones from mated and unmated females. However, Hegdekar and Dondale (1969) found no evidence of an olfactory pheromone in any of the four species tested.

For a number of reasons, *Pardosa milvina* males may rely more heavily on an airborne sex pheromone for communication relative to any other species

of wolf spider so far studied. First, *P. milvina* are widely ranging spiders and do not exhibit a sit-and-wait foraging strategy typical of most species of wolf spider, including other *Pardosa* spp. (Walker et al., 1999; Ford, 1978). Greater activity may make locating females through silk draglines more difficult and time consuming for males than through airborne pheromones. Second, greater movement of females would likely make draglines more costly to produce. Furthermore, since male *Pardosa* spp. commonly cover more distance than females in daily travel (Hallander, 1967), sole production of this pheromone by females is likely, although female response to airborne pheromones from other females or males was not examined in our study.

For our experiments, *P. milvina* were collected in soybean fields with open ground and little vegetation. Richter (1970) found a correlation between habitat structure of *Pardosa* spp. and the quantity of draglines produced. He found that species collected from open habitats and short vegetation tended to produce smaller quantities of silk as they moved through the environment than species from taller, more dense vegetation. Based on his findings, our population of *P. milvina* should produce less silk, which would increase the benefits of using an airborne pheromone in conjunction with a contact sex pheromone to facilitate communication between the sexes. Although laboratory observations indicate that *P. milvina* females do not produce large amounts of silk, further studies on *P. milvina* need to be conducted in these areas to understand the relative importance of contact versus airborne pheromones.

Male *P. milvina* showed a significant lack of response (neither positive nor negative) in the presence of an airborne chemical from immature females as well as adult males, and this effect increased when two males were used. Ayyagari and Tietjen (1986) found that male *Schizocosa ocreata* produce a male contact pheromone that inhibits male courtship and induces immobility in other males. Such a pheromone is sufficient to prevent courtship behavior when presented in high concentrations even in the presence of female contact sex pheromones. Our results suggest that an airborne inhibitory pheromone is produced by male and possibly subadult female *P. milvina*.

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SEX PHEROMONE COMPONENTS OF CASUARINA MOTH, *Lymantria xylina*

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Abstract—*cis*-7,8-Epoxy-2-methyleicosane is a sex pheromone component of the Casuarina moth, *Lymantria xylina* Swinhoe. The compound was extracted from pheromone glands of female moths and was identified by coupled gas chromatographic–electroantennographic detection (GC-EAD) and GC–mass spectrometry. In field experiments in Taiwan, traps baited with either or both of (7*R*,8*S*)-*cis*-7,8-epoxy-2-methyleicosane (>99% ee) [termed here (+)-xylinalure] and (7*S*,8*R*)-*cis*-7,8-epoxy-2-methyleicosane (>99% ee) [termed here (−)-xylinalure] captured male *L. xylina*. Addition of further candidate pheromone components to xylinalure did not enhance its attractiveness. Demonstration of whether or not female *L. xylina* produce both optical isomers of xylinalure, and determination of the ratio, will require pheromone extract analyses on a chiral, enantiomer-separating column (as yet unavailable) or derivatization of epoxides in accumulated gland extracts. Attraction of male *L. xylina* to either enantiomer of xylinalure contrasts with enantiospecific production of, and/or response to, epoxy pheromones in congeners. With no other nocturnal lymantriid moth known in Taiwan to utilize xylinalure for pheromonal communication, enantiospecific "fine tuning" of xylinalure, or evolution of a more complex pheromone blend, may not have been neces-

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sary for *L. xylina* to maintain specificity of sexual communication. Racemic xylinalure will be appropriate for pheromone-based detection surveys of *L. xylina* in North America.

Key Words—Lepidoptera, Lymantriidae, *Lymantria xylina*, *Lymantria dispar*, *Lymantria monacha*, *Lymantria fumida*, sex pheromone, reproductive isolation, (7*R*,8*S*)-*cis*-7,8-epoxy-2-methyleicosane, (7*S*,8R)-*cis*-7,8-epoxy-2-methyleicosane, 2-methyl-*Z*7-eicosene, (7*R*,8*S*)-*cis*-7,8-epoxy-2-methylnonadecane, (7*S*,8*R*)-*cis*-7,8-epoxy-2-methylnonadecane, (7*R*,8*S*)-*cis*-7,8-epoxy-3-methylnonadecane, (7*S*,8*R*)-*cis*-7,8-epoxy-3-methylnonadecane, disparlure.

INTRODUCTION

The Casuarina moth, *Lymantria xylina* Swinhoe (Lepidoptera: Lymantriidae), formerly known as *Lymantria sakaguchii* Matsumura (Kishida, 1995), occurs in southern Japan (Kyushu, Okinawa, and islands between), Taiwan, and southeastern China (Inoue, 1957; Li et al., 1981; Xiao, 1992). Caterpillars feed on many hardwood trees, especially *Casuarina* spp. (Li et al., 1981; Chao et al., 1996). Outbreak populations have completely defoliated host trees, especially *Casuarina*, on both sides of the Taiwan Straits (Sonan, 1936; Li et al., 1981; Chang and Weng, 1985; Xiao, 1992; Schaefer and Gries, unpublished data).

Pheromone-based detection surveys in North America offer the best means of early detection and eradication of invading Eurasian forest defoliators, such as *L. xylina*. Yet, the sex pheromone of *L. xylina* has not been thoroughly investigated. Y.-S. Chou (1982, unpublished data) captured limited numbers of *L. xylina* males in traps baited with disparlure (*cis*-7,8-epoxy-2-methyloctadecane), the sex pheromone of the gypsy moth, *Lymantria dispar* L. (Bierl et al., 1970). Similarly, P. W. Schaefer succeeded in 1994, but not in 1996 and 1997, in capturing male *L. xylina* in milk-carton traps (Ecogen Inc., Langhorne, Pennsylvania) baited with (+)-disparlure [1994: laminate bait type (Hercon Environmental Company, Emigsville, Pennsylvania); 1996–1997: string bait type (Phero Tech Inc., Delta, British Columbia, Canada)]. We report identification and field testing of sex pheromone components of *L. xylina*.

METHODS AND MATERIALS

Insect Culture and Pheromone Extraction. On May 23, 1997, *L. xylina* pupae were collected in coastal plantings of *Casuarina equisetifolia* L. in Kuanyin, Taoyuan County, Taiwan, and shipped to the Biological Control Laboratory, Taiwan National University in Taipei. Female pupae (Xiao, 1992) were kept in individual plastic cups at a photoperiod of 14L : 10D, 24–26°C, and 60–80% relative humidity, whereas male pupae were kept at ~15°C to retard development. Abdominal tips with pheromone glands of calling, 1- to 3-day-old

virgin female moths were removed and placed in redistilled hexane. After 15–30 min of extraction, the supernatant was withdrawn, syringed into ampoules, and shipped (together with male pupae) to Simon Fraser University.

Laboratory Analyses and General Procedures. Aliquots of 1 female equivalent (FE) of pheromone gland extract were analyzed by coupled gas chromatographic–electroantennographic detection (GC-EAD) (Arn et al., 1975), by using a Hewlett-Packard (HP) 5890A gas chromatograph equipped with a fused silica column (30 m × 0.25 or 0.32 mm ID) coated with either DB-210, DB-5, or DB 23 (J&W Scientific, Folsom, California). GC–mass spectrometry (MS) of synthetic or insect-produced compounds in full-scan electron ionization mode employed a Varian Saturn II Ion Trap GC-MS fitted with the DB-5 column referred to above. Nuclear magnetic resonance (NMR) spectroscopy of synthetic compounds was conducted on a Bruker AMX-400 spectrometer at 400.13 MHz for ^{1}H NMR spectra. ^{1}H chemical shifts are reported as parts per million (ppm) relative to TMS (0.00 ppm). Column chromatography refers to flash chromatography that used silica gel 60 (230–400 mesh, E. Merck, Darmstadt) (Still et al., 1978).

Synthesis of 2-Methyl-Z7-eicosene (4) and cis-*7,8-Epoxy-2-methyleicosane (5) (Figure 1).* Coupling of 40 ml (166.7 mmol) of dodecylbromide with the dianion of 5-hexyne-1-ol (**1**; 10 g, 102 mmol) in the presence of HMPA, and subsequent hydrogenation (Ni-P2 catalyst) of 5-octadecyn-1-ol (**2**) afforded (*Z*)-5-octadecen-1-ol (**3**) (25.5 g, 95 mmol, 93% yield). Mesylation at 0°C of **3** with methanesulfonyl chloride (100 mmol) in the presence of triethylamine (150 mmol) and subsequent copper(I)-catalyzed (10 mmol of CuI) coupling of mesylate with 2-bromomagnesium propane (190 mmol) at −25°C produced 2-methyl-*Z*7-eicosene (**4**). Column chromatography of the crude product with hexanes and

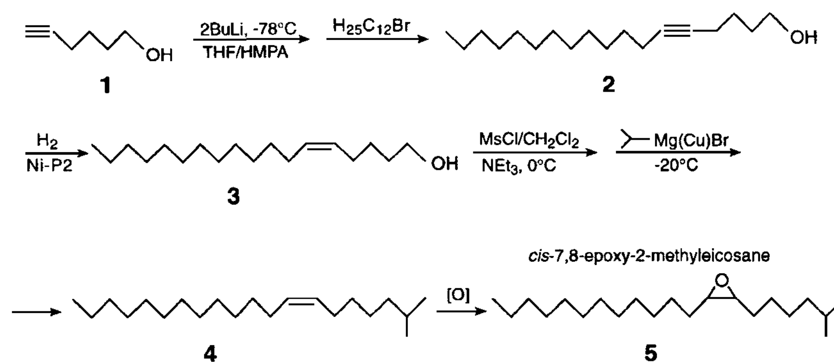

FIG. 1. Schemes for synthesis of 2-methyl-*Z*7-eicosene and *cis*-7,8-epoxy-2-methyleicosane.

then hexanes–ether (4 : 1) as eluents allowed purification of hydrocarbon **4** (8.48 g, 30% yield) and recovery of starting alcohol **3** (13 g, 51%), which was used for repeated mesylate–Grignard coupling. Combined yields of **4** were 19.28 g (69% yield based on alcohol **3**). Stirring **4** (10.8 g, 36.7 mmol) with 1.5 molar excess of *m*-chloroperoxybenzoic acid in dichloromethane at 0°C for 3 hr, and then overnight at room temperature, afforded *cis*-7,8-epoxy-2-methyleicosane (**5**) (10.82 g, 95% yield after purification by column chromatography that used 2% of ether in hexanes as the eluent). ^{1}H NMR of **5**: 2.90 (quint, 2H), 1.49 (m, 4H), 1.15–1.42 (m, 27H), 0.88 (t, 3H), 0.87 (d, 6H).

Synthetic standards of *cis*-7,8-epoxy-2-methylnonadecane; *cis*-7,8-epoxy-3-methylnonadecane, (7*R*,8*S*)-*cis*-7,8-epoxy-3-methylnonadecane (*cis*-7*R*,8*S*-epoxy-3me-19Hy), and (7*S*,8*R*)-*cis*-7,8-epoxy-3-methylnonadecane (*cis*-7*S*,8*R*-epoxy-3me-19Hy), were available from previous work (Gries et al., 1996). Enantiospecific syntheses of (7*R*,8*S*)-*cis*-7,8-epoxy-2-methylnonadecane (*cis*-7*R*,8*S*-epoxy-2me-19Hy), (7*S*,8*R*)-*cis*-7,8-epoxy-2-methylnonadecane (*cis*-7*S*,8*R*-epoxy-2me-19Hy), (7*R*,8*S*)-*cis*-7,8-epoxy-2-methyleicosane (*cis*-7*R*,8*S*-epoxy-2me-20Hy) and (7*S*,8*R*)-*cis*-7,8-epoxy-2-methyleicosane (*cis*-7*S*,8*R*-epoxy-2me-20Hy) followed methods previously developed by R. Hahn.

Field Experiments. Field experiments were conducted in several coastal plantings of *C. equisetifolia* in Kuanyin and Taoyuan, Taoyuan County, Taiwan. Self-made sticky 2-liter Delta milk carton traps (Gray et al., 1984) were suspended from trees ~2 m above ground in complete randomized blocks with trap spacings of 15–20 m. Traps were baited with gray sleeve stoppers (The West Company, Lionville, Pennsylvania) impregnated with test chemicals in HPLC grade hexane. Experiments 1–4 tested the major candidate pheromone components *cis*-7*R*,8*S*-epoxy-2me-20Hy and *cis*-7*S*,8*R*-epoxy-2me-20Hy singly and in combination, each at 50 μg (experiment 1), 5 μg (experiment 2), 0.5 μg (experiment 3), and 0.05 μg (experiment 4). Experiments 5–7 tested *cis*-7*R*,8*S*-epoxy-2me-20Hy (50 μg) singly and in combination with additional candidate pheromone components, such as either and both of: *cis*-7*R*,8*S*-epoxy-2me-19Hy (5 μg) and *cis*-7*S*,8*R*-epoxy-2me-19Hy (5 μg) (experiment 5); *cis*-7*R*,8*S*-epoxy-3me-19Hy (5 μg), and *cis*-7*S*,8*R*-epoxy-3me-19Hy (5 μg) (experiment 6); and with 2-methy-*Z*7–20Hy at 0.5, 5, and 50 μg. Experiment 8 tested *cis*-7*R*,8*S*-epoxy-2me-20Hy plus *cis*-7*S*,8*R*-epoxy-2me-20Hy at increasing doses (5, 50, and 500 μg each). Final experiment 9 tested either *cis*-7*R*,8*S*-epoxy-2me-20Hy (500 μg) plus *cis*-7*S*,8*R*-epoxy-2me-20Hy (500 μg) or *cis*-7*R*,8*S*-epoxy-2me-18Hy [(+)-disparlure; 500 μg)] alone or in combination.

RESULTS AND DISCUSSION

GC-EAD analyses of pheromone gland extract of female *L. xylina* revealed four compounds that consistently elicited responses from male moth antennae

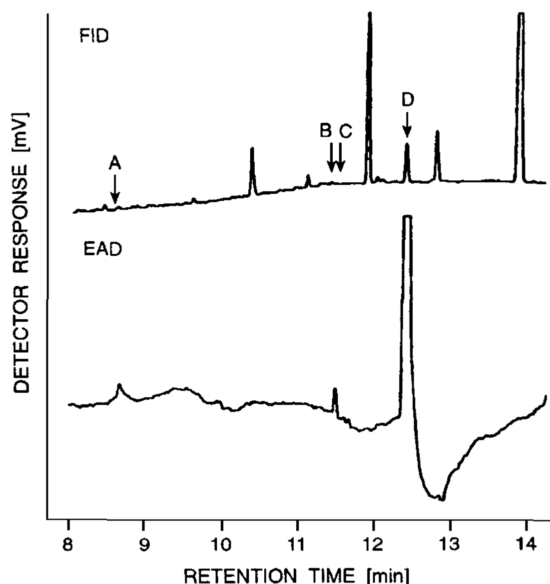

FIG. 2. Flame ionization detector (FID) and electroantennographic detector (EAD: male *L. xylina* antenna) responses to 1 FE of pheromone extract. Hewlett-Packard 5890A gas chromatograph equipped with a fused silica column (30 m × 0.25 mm ID) coated with DB-23: temperature program: 100°C (1 min), 10°C/min to 200°C.

(Figure 2). Most abundant and most EAD-active compound **D** had retention indices (Dool and Kratz, 1963) ca. 200 units higher than those of disparlure on all three analytical columns (DB-5, DB-210, and DB-23), indicative of an epoxide homologous to disparlure. The mass spectrum of **D** with molecular ion m/z 311 (M+1) and fragmentation ion m/z 211 (Figure 3) suggested *cis*-7,8-epoxy-2me-20Hy as a potential molecular structure. Corresponding retention times and mass spectra as well as comparable antennal activity of insect-produced **D** and synthetic *cis*-7,8-epoxy-2me-20Hy support the structural assignment. Moreover, mass spectra of synthetic *cis*-6,7- or *cis*-8,9-epoxy-2me-20Hy were inconsistent with insect-produced **D**. EAD-active compound **A** with retention indices 200 units higher than those of the olefin analog of disparlure (2-methyl-Z7-octadecene) was hypothesized and, through comparative GC-EAD on all three columns, confirmed to be 2-methyl-Z7-eicosene. EAD-active compound **B** (not visible in Figure 2) cochromatographed on all employed analytical columns (DB-5, DB-210, and DB-23) with synthetic *cis*-7,8-epoxy-3-methylnonadecane (Gries et al., 1996). EAD-active compound **C** with retention indices on all three columns consistently ca. 100 units lower than that of **D** was hypoth-

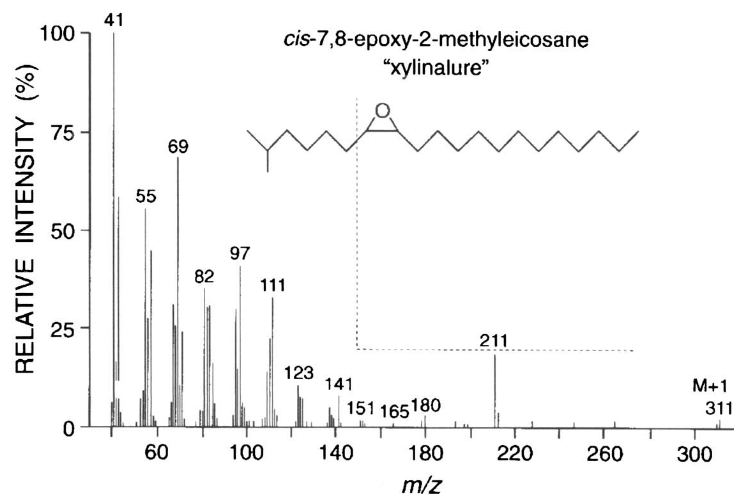

FIG. 3. Full-scan electron ionization mass spectrum of *cis*-7,8-epoxy-2-methyleicosane (xylinalure) present in pheromone gland extracts of female *L. xylina*; Varian Saturn II Ion Trap GC-MS.

esized and, through comparative GC-EAD with an authentic standard, confirmed to be *cis*-7,8-epoxy-2me-19Hy.

In field experiments (Figure 4, experiments 1–4), traps baited with either or both *cis*-7*R*,8*S*-epoxy-2me-20Hy [termed here (+)-xylinalure] and *cis*-7*S*,8*R*-epoxy-2me-20Hy [termed here (−)-xylinalure] captured male *L. xylina*. Other candidate pheromone components, such as *cis*-7*R*,8*S*-epoxy-2me-19Hy and/or *cis*-7*S*,8*R*-epoxy-2me-19Hy (Figure 5, experiment 5), *cis*-7*R*,8*S*-epoxy-3me-19Hy and/or *cis*-7*S*,8*R*-epoxy-3me-19Hy (Figure 5, expriment 6), or 2-methyl-*Z*7-20Hy (Figure 5, experiment 7) neither enhanced nor reduced attractiveness of xylinalure, indicating that they are not part of the pheromonal blend.

Variation in relative attractiveness of (+)-, (−)-, and (+)- plus (−)-xylinalure in experiments 1–4 (Figure 4) may be explained, in part, by the heterogeneity of trapping sites, particularly those of experiments 2 and 3. Furthermore, low pheromone doses in experiments 2–4, attracting male moths mainly in the first night of trapping, possibly coupled with low emergence of new males in subsequent nights, may have prevented normalization of skewed trapping results. As demonstrated in the leaf-mining moth *Eriocrania semipurpurella* (Löfstedt et al., 1998), it is also possible that genetically related subsets of male *L. xylina* exhibited preference for (+)-, (−)-, or (+)- plus (−)- pheromone enantiomers in temporally and/or spatially separated experiments 1–4.

With the similar attractiveness of (+)- and (−)-xylinalure, even at lure load-

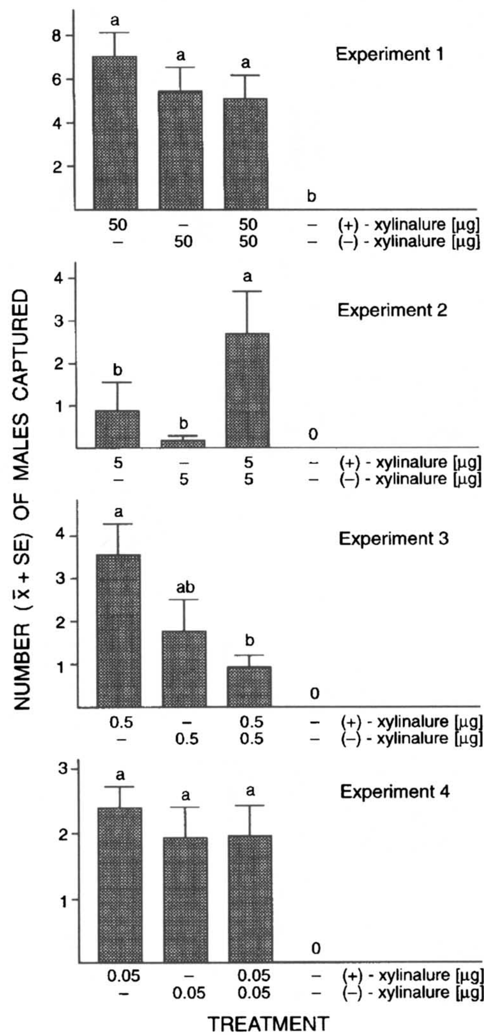

FIG. 4. Mean number (+SE) of male *L. xylina* captured in sticky 2-liter Delta milk carton traps baited with either or both (+)-xylinalure [(7*R*,8*S*)-*cis*-7,8-epoxy-2-methyleicosane] and (−)-xylinalure [(7*S*,8*R*)-*cis*-7,8-epoxy-2-methyleicosane], each at 50 μg (experiment 1), 5 μg (experiment 2), 0.5 μg (experiment 3), and 0.05 μg (experiment 4); 10 replicates per experiment; May 20–21, 1998 (experiment 1), May 22–24, 1998 (experiments 2 and 3), May 23–24, 1998 (experiment 4); near Kuanyin and Ta yuan, Taoyuan County, Taiwan. For each experiment, bars with the same letter are not significantly different; nonparametric analysis of variance by ranks (Friedman's test) followed by comparison of means [Bonferroni *t* test, $P < 0.05$] (SAS/STAT, 1988).

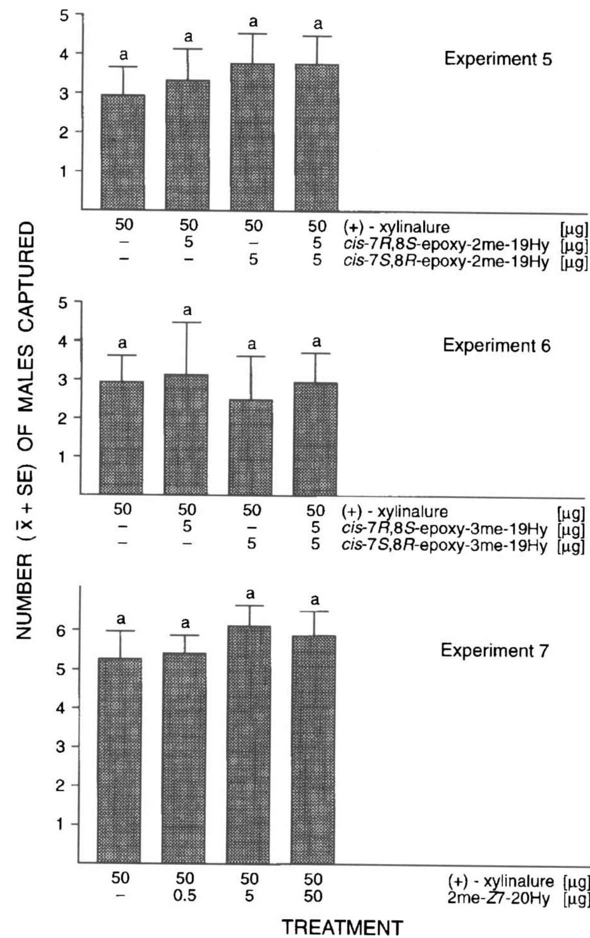

FIG. 5. Mean number (+SE) of male *L. xylina* captured in sticky 2-liter Delta milk carton traps baited with (+)-xylinalure [(7*R*,8*S*)-*cis*-7,8-epoxy-2-methyleicosane] singly and in combination with additional candidate pheromone components, such as either or both (7*R*,8*S*)-*cis*-7,8-epoxy-2-methylnonadecane (*cis*-7*R*,8*S*-epoxy-2me-19Hy) and (7*S*,8*R*)-*cis*-7,8-epoxy-2-methylnonadecane (*cis*-7*S*,8*R*-epoxy-2me-19Hy) (experiment 5), either or both (7*R*,8*S*)-*cis*-7,8-epoxy-3-methylnonadecane (*cis*-7*R*,8*S*-epoxy-3me-19Hy) and (7*S*,8*R*)-*cis*-7,8-epoxy-3-methylnonadecane (*cis*-7*S*,8*R*-epoxy-3me-19Hy) (experiment 6), and with 2-methyl-*Z*7-eicosene (2me-*Z*7-20Hy); 10 replicates per experiment; May 21–22, 1998; near Kuanyin and Taoyuan, Taoyuan Company, Taiwan. For each experiment, there were no significant differences between treatments; nonparametric analysis of variance by ranks (Friedman's test) followed by comparison of means [Bonferroni *t* test, $P > 0.05$] (SAS/STAT, 1988).

ings as low as 0.05 μg (Figure 4, experiment 4), and enantiomeric purities of test chemicals exceeding 99% ee (Hahn et al., unpublished), enantiomeric contamination could not have been responsible for the behavioral activity of either enantiomer. Demonstration whether or not female *L. xylina* produce both optical isomers of xylinalure, and determination of the ratio, will require GC-EAD and GC-MS analyses of pheromone extract on a chiral, enantiomer-separating column (as yet unavailable) or derivatization of epoxides (Oliver and Waters, 1995) in accumulated gland extracts.

Attractiveness of either enantiomer of xylinalure in *L. xylina* (Figure 4, experiments 1–4) contrasts with enantiospecific attractiveness of (+)-disparlure in the closely related gypsy moth, *L. dispar.* (+)-Disparlure attracts male *L. dispar*, whereas the presence of (−)-disparlure reduces attractiveness of the bait (Cardé et al., 1977a,b; Miller et al., 1977; Plimmer et al., 1977). The presence of 2-methyl-*Z*7-octadecene (the olefin analog of disparlure) also reduces attractiveness of disparlure to male *L. dispar* (Cardé et al., 1973; Miller et al., 1977), whereas analogous 2-methyl-*Z*7-eicosene in *L. xylina* has no behavioral effect.

With at least three sympatric species of lymantriid moths that utilize disparlure as a pheromone component, including *L. dispar* (Bierl et al., 1970); the nun moth, *L. monacha* L. (Bierl et al., 1975); and *L. fumida* (Schaefer et al., unpublished), enantiospecificity in the production of (Hansen, 1984) and/or response to disparlure may have evolved to contribute to species-specific sexual communication. 2-Methyl-*Z*7-octadecene, serving as a pheromone component in both *L. monacha* (Grant et al., 1996; Gries et al., 1996) and *L. fumida* (Schaefer et al., 1999), but being repellent to male *L. dispar*, seems to enhance specificity of communication channels. Xylinalure, in contrast, is reported here for the first time as a pheromone component in lymantriid moths. With no other nocturnal lymantriids in Taiwan known to utilize xylinalure for pheromonal communication, enantiospecific fine tuning of xylinalure, or evolution of a more complex pheromone blend, may not have been necessary for *L. xylina* to maintain reproductive isolation.

The (almost) indiscriminate response by male *L. xylina* to (+)-xylinalure with or without (+)-disparlure (Figure 6, experiment 9) may be attributed to the absence of *L. dispar* in Taiwan. Whether sympatry and coseasonality of *L. xylina* and *L. dispar* on other islands, such as Okinawa, alter the response to heterospecific pheromone and/or diel periodicity of pheromonal communication is currently being investigated. This research has implications for pheromone-based detection surveys for Asian lymantriids in North America, as it will facilitate decisions whether pheromones of two or more lymantriids, such as *L. xylina* and *L. dispar*, can be economically combined in one lure without compromising optimal attraction of all target species.

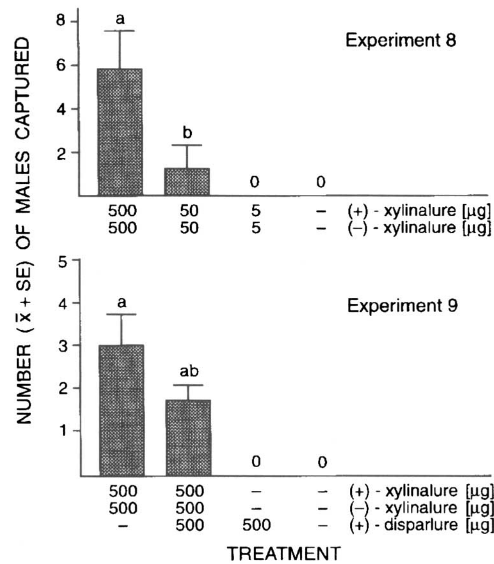

FIG. 6. Mean number (+SE) of male *L. xylina* captured in sticky 2-liter Delta milk carton traps baited with various doses of (+)- and (−)-xylinalure [(7*R*,8*S*)- and (7*S*,8*R*)-*cis*-7,8-epoxy-2-methyleicosane] (experiment 8), and (+)- plus (−)-xylinalure or (+)-disparlure [(7*R*,8*S*)-*cis*-7,8-epoxy-2-methyloctadecane) singly and in combination (experiment 9); 10 replicates for each experiment; May 25–26, 1998; near Kuanyin and Taoyuan, Taoyuan Company, Taiwan. For each experiment, bars with the same letter are not significantly different; nonparametric analysis of variance by ranks (Friedman's test) followed by comparison of means [Bonferroni *t* test, $P > 0.05$] (SAS/STAT, 1988).

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A NOVEL BIOASSAY FOR *Yponomeuta cagnagellus* OVIPOSITION IN RESPONSE TO EXTRACTS OF HOST AND NONHOST PLANT SURFACE COMPOUNDS

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Abstract—*Yponomeuta cagnagellus* is a phytophagous moth species specialized on *Euonymus europaeus*. Host discrimination by the adult female is an important aspect of host specialization and is based mainly on the distinctive secondary chemistry of host and nonhosts. This paper describes a bioassay that was developed to study the effect of isolated plant surface compounds on *Yponomeuta* oviposition. Adult moths recognize their hosts through chemical stimuli on the leaf or twig surface. Relatively apolar compounds extracted from the host twig surface by washing in dichlormethane do not stimulate oviposition. More polar, methanol-soluble compounds do, and this stimulation is dose dependent. Moths are able to recognize hosts solely by their surface compounds: females show a strong preference for artificial twigs treated with methanolic extracts of their hosts compared to those treated with methanolic extracts of nonhosts *Crataegus monogyna* and *Prunus spinosa* (both of which are hosts for closely related *Y. padellus*). Shape and surface characteristics of the oviposition substrate also influence oviposition. The substrate needs to resemble the basic form of a twig (i.e., cylindrical), and females prefer a coarse surface with irregularities over a smooth one.

Key Words—*Yponomeuta cagnagellus*, ermine moth, Lepidoptera, speciation, specialization, plant surface compounds, oviposition, host discrimination, *Euonymus europaeus*.

INTRODUCTION

The modification of host acceptance behavior and oviposition in particular can be viewed as the key to evolutionary modifications of host range in phytophagous

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insects (Dethier, 1982; Berenbaum, 1990; Bush, 1994; Menken and Roessingh, 1998). In lepidopteran species specialized on one or a few host plants, the correct choice by the adult female is critical, as the larvae generally will not readily feed on other plant species. Moreover, considering the small size of most first-instar larvae, they are not likely to find an acceptable food source without large fitness costs in the event of oviposition mistakes. However, the female is not able to assess the nutritional quality of her offspring's food plant directly, as she is unable to feed on it and is, thus, dependent on indirect cues present on the stem and leaf surface for host recognition.

Yponomeuta species (Lepidoptera: Yponomeutidae) form a genus of highly specialized phytophagous moths. Food plant associations are known in 38 of 70 species within this genus (exclusive of the American species): 31 species are monophagous on host plants belonging to one genus. Of these, 23 are found on plants in *Euonymus* (Celastraceae). Of the nine European *Yponomeuta* species, only three occur on a celastraceaous host (*Euonymus europaeus* L.), the other six are specialized on plants belonging to the Rosaceae, Salicaceae, or Crassulaceae (Gershenson and Ulenberg, 1998). Their phylogeny indicates that shifts from an ancestral celastraceous host to other hosts—mainly rosaceous—may have been responsible for the evolution of the European species (Menken et al., 1992; Menken and Roessingh, 1998).

Currently, the potential for a change in the discriminative behavior of the adult *Yponomeuta* species and its possible contribution to the occurrence of host shifts and subsequent host race formation and speciation is being studied. As a starting point for understanding this behavior, identification of the specific plant compounds involved in adult host recognition is necessary. Therefore, in this study, we focus on the response of gravid females to plant surface extracts, asking whether *Yponomeuta cagnagellus* (Hbn.) is capable of recognizing the host from compounds available on the plant surface. An efficient tool for the assessment of *Yponomeuta* oviposition in response to chemical compounds was developed and used to evaluate stimulatory activity of extracts of plant surfaces washed with organic solvents.

METHODS AND MATERIALS

Insects. Yponomeuta cagnagellus moths were reared from larvae fed leaves of the host plant *Euonymus europaeus* in a climate room of 18°C or 23°C, 17L : 7D. Caterpillars were collected in the field from host plants in June–July as fourth or fifth instars (Amsterdam), or in February as diapausing first instars by collecting *E. europaeus* twigs with *Y. cagnagellus* hibernacula (The Hague, Meyendel). The latter were stored at 5°C in complete darkness until use. After pupation, the pupae were placed individually in glass vials. Newly emerged

moths were fed with a solution of 10 g honey in 1 liter of 1% water–agar that was provided in 0.5 ml Eppendorf tubes every three to four days. *Yponomeuta cagnagellus* females take up to 14 days to become sexually mature (Hendrikse, 1979). To obtain gravid insects, individual females were provided with a mate and kept at 18°C, 17L : 7D for two weeks after emergence before using them in the oviposition experiments.

Bioassay. Artificial twigs (ATs) (Figure 1) were constructed from glass Pasteur capillary pipets (WU, Mainz, Germany). The diameter of these (70 mm) is similar to *E. europaeus* twigs with egg masses in the field. *Yponomeuta cagnagellus* preferably position their egg masses near buds, nodes, and other irregularities on the twig surface (Bremner et al., 1997). To simulate these structures, two orthodontic rubber bands (64 mm diameter) were placed on the pipets. For some experiments, the glass pipet was covered with paper adhesive tape (TESA, Beiersdorf BDF).

The ATs were sprayed with various plant extracts and solvents by using a compressed air reagents diffuser. To obtain a homogenous distribution of the

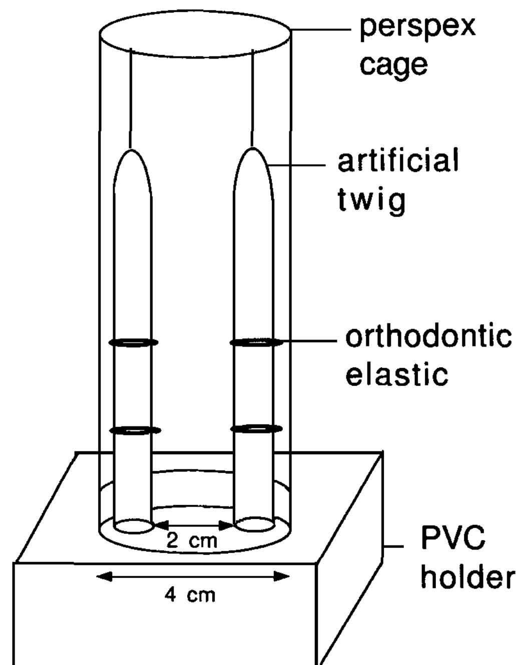

FIG. 1. Model of the cage and artificial twigs used for oviposition preference experiments.

extracts on the ATs, 5–15 pipets were fixed side-by-side on a cardboard strip, thus presenting a more-or-less two-dimensional surface. Since amounts of extract were limited, only the lower 5 cm were treated, excluding the first 1 cm by which the ATs were fixed in their holders. The ATs were mounted in PVC or polystyrene holders and placed in cages. In two-choice bioassays, one female moth was added to the Perspex cage (4 cm diam., height 15 cm; Figure 1) accompanied by her mate. For bioassays presenting a choice among more than two treatments, 5–10 pairs of moths were introduced in large cages of transparent plastic (70 cm high, 30 cm diam.) to obtain adequate amounts of egg masses for statistical analysis.

Yponomeuta cagnagellus deposits an egg mass once every two to three days, resulting usually in one to four and maximally 10 egg masses. Therefore, the moths were allowed to oviposit for their remaining life-spans (three to four weeks). During this period, they were fed with a solution of 10 g honey in 1 liter of 1% water–agar supplied in 0.5 ml Eppendorf tubes. The egg masses laid in the bioassays were counted every two to four days. Behavioral observations on *Y. cagnagellus* have shown that the final choice of the oviposition site is made before the actual egg deposition. During the deposition of the egg mass, the host is no longer examined (Hora and Roessingh, 1999). Therefore, preference was determined from the number of egg masses laid on each alternative rather than on the total number of eggs.

Plant Extracts. ATs were treated with phytochemicals extracted from the twig or leaf surface of host plant *E. europaeus*, or of non-hosts *C. monogyna* (Jacq.) and *P. spinosa* L. The latter two are hosts for the closely related oligophagous *Y. padellus* L. but are not accepted for oviposition by *Y. cagnagellus* (Bremner et al., 1997).

Surface extracts were prepared as described by Städler and Roessingh (1991). Approximately 8 m of leafless twigs, cut into 10-cm pieces, or intact leaves were dipped for 5 sec in 0.5 l CH_2Cl_2 followed by two 5 sec dips in two portions of 1 l MeOH. To enable calculation of the ensuing concentration of extract, the diameter (d) and length (l) of the twigs were measured, and the total surface of the extracted twigs was calculated as $\Sigma(l * \pi * d)$. For the leaf surface extract, the total leaf area was measured by using a photoplanimeter. The concentration of the extracts was expressed in twig (or leaf) surface equivalents (TSE or LSE), with units of square centimeters per milliliter of solvent. Concentration of the extract on the AT was controlled by application of volumes of the extract in proportion to the surface treated. Thus, 1 TSE on the AT equaled the concentration of compounds extracted from a similar twig surface.

Fractions were concentrated in a rotary evaporator under reduced pressure of approximately 190 mbar at a temperature of 40°C to a volume of approximately 40 ml. Methanolic extracts were pooled before evaporation. Thus, two fractions were obtained: the methanolic fraction containing the polar phyto-

chemicals: and the dicholormethane (CH_2Cl_2) fraction containing the more apolar substances.

Experiments with Surface Extracts of E. europaeus. A first series of experiments served to examine the stimulatory effect of surface extracts of the host *E. europaeus*. The ATs in these experiments consisted of plain glass pipets. The complete extract (i.e., a mixture of the methanol and CH_2Cl_2 fraction) was applied. The methanol fraction was applied first and allowed to dry, whereupon the CH_2Cl_2 fraction was added. The treated ATs were offered in two-choice experiments. The alternative choices were either a host plant twig (of equal length and diameter to the AT and kept fresh in 1% water agar) or a clean glass control (consisting of an AT treated with the solvents only). Two repetitions of 10 and eight individual females each in both choice situations were carried out. The concentration of the twig surface extracts was 1 TSE for the first trial and 0.1 TSE for the second. The results of the repetitions were pooled since no significant differences were found (Mann-Whitney, $P > 0.05$).

To determine which fraction contained compounds stimulating oviposition, the methanol and the CH_2Cl_2 fractions were applied separately on the artificial twigs. ATs treated with the two separate fractions were offered to individual females in two-choice tests ($N = 28$). Additionally, ATs treated with the methanolic extract were compared with either an *E. europaeus* twig ($N = 23$) or a control pipet treated with methanol ($N = 29$). Two repetitions were carried out with concentrations of 1 TSE.

The methanolic extract of host plant twigs was tested against the methanolic extract of leaves to determine whether the moths could discriminate between leaves and twigs when only phytochemical information is present. Both extracts were applied on ATs in a concentration of 1 TSE for the twig surface extract and 1 LSE for the leaf surface extract. Both were offered in a two-choice situation to 26 individual female moths.

The response of females to different concentrations of the methanolic twig surface fraction was determined by simultaneously offering six ATs treated with increasing concentrations of the extract to groups of 10 pairs of moths. A first series of experiments was carried out comparing concentrations of 0.0, 1×10^{-3}, 1×10^{-2}, 1×10^{-1}, 5×10^{-1}, and 1 TSE. This comparison was repeated 12 times with a total of 120 females. In the second series, a higher concentration of 5 TSE was added to the exclusion of the 5×10^{-1} TSE concentration. This was repeated 9 times with 90 female moths. The various concentrations were prepared by diluting increasing volumes of the original extract with MeOH to 1.5 ml, which was then sprayed on 9–12 ATs.

Experiments with Twig Surface Extract of Host and Nonhosts. ATs covered with paper tape and treated with 1 TSE of methanolic *E. europaeus* or *C. monogyna* extract were offered in a two-choice situation to 80 female moths in eight cages with 10 females per cage. The same experiment was carried out with

P. spinosa extract as the alternative choice to extract of *E. europaeus* (25 females in five cages with five females per cage).

Form and Structure of Oviposition Site. This experiment was carried out to determine the effect of the basic cylindrical form of a twig. Sixteen gravid females were offered 1 TSE of methanolic *E. europeaus* extract on a piece of filter paper (Whatman, 4.2 cm diameter) in a no-choice situation. The filter paper was treated with an amount of extract corresponding to the surface area. A ridge was folded in the center to present an irregularity in the surface. The papers were offered in round plastic Petri dishes (10 cm diameter, 1.5 cm high). The amounts and location of egg masses laid by these females were compared to those of 50 females from the same rearing unit that had been preconditioned under the same circumstances and were presented with cylindrical ATs treated with 1 TSE of the same methanolic extract. The ATs were covered with paper adhesive tape to present a similar surface texture.

Surface texture was tested by comparing oviposition on glass or tape ATs. Fifty females were tested in five cages containing a choice between glass ATs and ATs covered with paper adhesive tape, both treated with 1 TSE of methanolic *E. europaeus* extract.

RESULTS

Surface Extracts of E. europaeus. The complete twig surface extract of *E. europaeus* was preferred over the clean glass control (Wilcoxon, $P < 0.001$; Figure 2). However, it was not as attractive as the natural host twig (Wilcoxon, $P < 0.05$; Figure 2). Moths preferred the MeOH fraction to the CH_2Cl_2 frac-

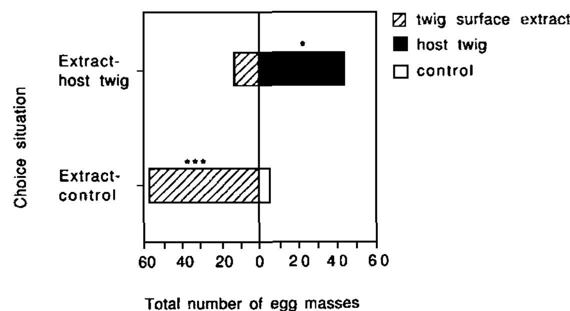

FIG. 2. Number of egg masses deposited by *Yponomeuta cagnagellus* in two-choice situations with artificial twigs treated with the complete twig surface extract of *Euonymus europeaus* and either cut twigs of *E. europaeus* (N = 18 females) or artificial twigs treated with solvents (N = 18 females) only. *Significantly higher number of egg masses, Wilcoxon, $P < 0.05$; ***Wilcoxon, $P < 0.001$.

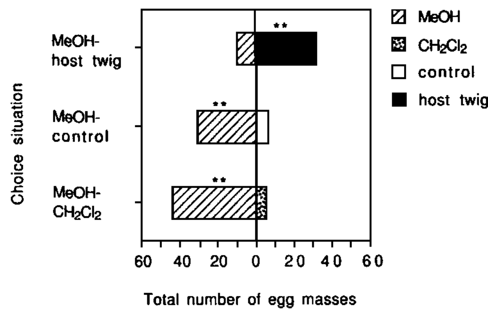

FIG. 3. Number of egg masses deposited by *Yponomeuta cagnagellus* in two-choice situations comparing artificial twigs treated with 1 TSE (twig surface equivalent) of the methanolic fraction of twig surface extract of *Euonymus europeaus* with cut twigs of *E. europaeus* ($N = 23$ females), with artificial twigs treated with solvent only ($N = 29$ females) or with 1 TSE of the CH_2Cl_2 fraction ($N = 28$ females). **Significantly higher number of egg masses, Wilcoxon, $P < 0.01$.

tion and the clean glass control (Wilcoxon, $P < 0.01$; Figure 3). Analogous to the complete extract, the MeOH fraction was not as attractive as the host twig (Wilcoxon, $P < 0.01$; Figure 3). We attempted to test the CH_2Cl_2 fraction against a negative control as well, but did not obtain an adequate level of oviposition; only one female of five deposited egg masses on the CH_2Cl_2 fraction, others did not oviposit at all.

The activity of the methanolic leaf surface extract did not differ from that of the twig surface extract (Wilcoxon, $P > 0.05$; Figure 4). The stimulatory activity of the twig surface extract was dose dependent (Figure 5). The highest number of egg masses was laid on the highest concentration of the extract in both repetitions. The two dose–response curves did not differ significantly (Kolmogornov-Smirnov, $P > 0.05$).

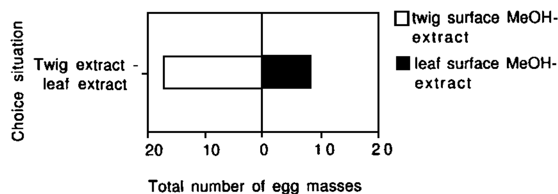

FIG. 4. Number of egg masses deposited by *Yponomeuta cagnagellus* in a two-choice situation with artificial twigs treated with either 1 LSE methanolic leaf surface extract or 1 TSE methanolic twig surface extract ($N = 26$ females). No significant difference was found (Wilcoxon, $P > 0.05$).

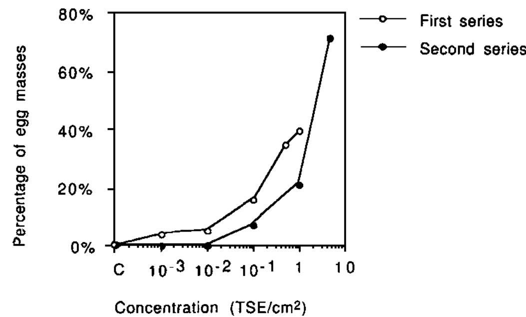

FIG. 5. Percentage of egg masses deposited by *Yponomeuta cagnagellus* in a six-choice situation with artificial twigs treated with increasing concentrations of methanolic twig surface extract. First series: 0.0, 1×10^{-3}, 1×10^{-2}, 1×10^{-1}, 5×10^{-1}, and 1 TSE ($N = 120$ females); Second series: 0.0, 1×10^{-3}, 1×10^{-2}, 1×10^{-1}, 1 TSE, and 5 TSE ($N = 90$ females).

Specificity of Twig Surface Extract. Yponomeuta cagnagellus preferred ATs treated with the twig surface extract of *E. europaeus* over ATs treated with either *C. monogyna* or *P. spinosa* extract (Wilcoxon, $P < 0.001$; Figure 6A and B).

Form and Structure of Oviposition Site. Of the 16 females, only four oviposited on the filter paper treated with the methanolic twig surface extract (on average 0.6 ± 0.3 egg mass/female). The others did not oviposit at all during the remainder of their life-spans. Of these four females, two also deposited

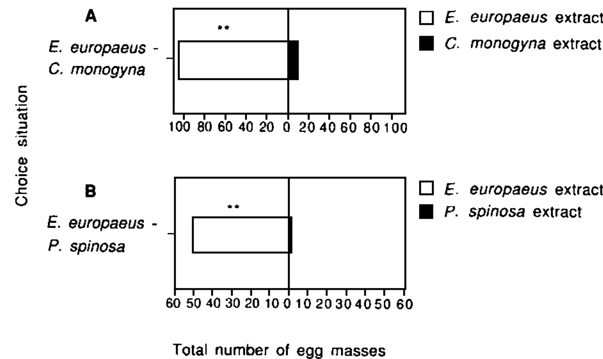

FIG. 6. Number of egg masses deposited by *Yponomeuta cagnagellus* in a two-choice situation with artificial twigs treated with 1 TSE of either methanolic twig surface extract of the host *Euonymus europaeus* or 1 TSE methanolic twig surface extract of non-host *Crataegus monogyna* (A; $N = 80$ females) or non-host *Prunus spinosa* (B; $N = 25$ females). **Significantly higher number of egg masses (Wilcoxon, $P < 0.01$).

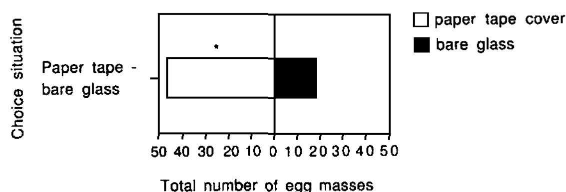

FIG. 7. Number of egg masses deposited by *Yponomeuta cagnagellus* in a two-choice situation with either bare glass artificial twigs or artificial twigs covered with paper tape, both treated with 1 TSE methanolic twig surface extract of *Euonymus europaeus* (N = 50 females). *Significantly higher number of egg masses (Wilcoxon, $P < 0.05$).

egg masses beside the filter papers on the floor of their Petri dishes (40% of all egg masses). The average number of egg masses laid by the control group on ATs was higher (1.5 ± 0.2 egg mass/female) (Mann/Whitney, $P < 0.05$). Moreover, deposition of egg masses on the floor of the cage, away from the AT in the control group, occurred only incidentally (9% of all egg masses). As in the preceding experiments, egg masses on the ATs were preferably deposited against the orthodontic elastics.

Tape-covered ATs were preferred over bare glass ATs (Wilcoxon, $P < 0.05$; Figure 7). The slight ridge that was formed by the paper tape where the ends meet presented an additional preferred oviposition site.

DISCUSSION

Surface Extracts. The results show that oviposition of *Y. cagnagellus* is stimulated by polar, MeOH-soluble compounds that can be washed from the surface of *E. europaeus* twigs (Figure 3). The stimulatory activity of this extract is dose dependent: higher concentrations increase oviposition (Figure 5). Plant surface extracts have been described to be effective oviposition stimulants in a number of insects (e.g., Städler and Roessingh, 1991). In butterflies, it seems to be a general characteristic that stimulant surface compounds are quite polar (Renwick and Chew, 1994). The compounds in the *E. europaeus* extract stimulating oviposition in *Y. cagnagellus* conform to this pattern.

In moths, the role of surface compounds has not been as well studied as for butterflies, although reports of the effectiveness of host plant homogenates or compounds distilled from these homogenates are available for some moths (references in Renwick and Chew, 1994; Honda, 1995). However, homogenates are not necessarily representative of the chemical composition on the actual plant surface. The relative concentration of compounds in leaf tissue is not always the same as on the surface, and some secondary compounds may not penetrate

to the surface (Soldaat et al., 1996). Therefore, the reaction of moths to plant homogenates might not represent their natural behavior.

We believe a better approach for testing behavioral responses is the use of extracts of the plant surface as described in this paper. However, there are only a few reports of oviposition of moths in response to plant surface extracts [e.g., *Acrolepiopsis assectella* (Thibout and Auger, 1996), *Keiferia lycopersicella* (Burton and Schuster, 1981), *Manduca sexta* (Bordner et al., 1983), *Ostrinia nubilalis* (Derridj et al., 1992)]. The extracts eliciting positive responses in these moths were obtained by using water, ethanol, or methanol solvents. *Acrolepiopsis assectella* and *K. lycopersicella*, both specialist moth species, showed oviposition responses similar to *Y. cagnagellus*, i.e., substrates treated with host plant extracts were preferred strongly over controls.

Although *Y. cagnagellus* showed a strong response to the surface extract of its host, the artificial twig treated with host surface extract was not as attractive as the real host plant twig (Figures 2 and 3). This might be a result of a combination of deficiencies of the artificial substrate. First, the extraction method might not extract all relevant surface components, and their concentrations might not be similar to those on the twig surface. An indication that the latter might be involved was present in the dose–response test, where a concentration of 5 TSE was preferred over 1 TSE (Figure 5). However, gravid *Y. cagnagellus* did not behave differently on natural *E. europaeus* twigs or artificial twigs treated with 1 TSE methanolic twig surface extract (Hora and Roessingh, 1999), implying that all relevant chemical cues are present in the extract. Second, the host twig may emit general, possibly stimulatory cues—such as moisture—which can enhance attractivity of oviposition substrates (Renwick and Chew, 1994). Visual and tactile cues may also modify oviposition behavior in moths (Renwick and Chew, 1994) and contribute to the preference for real twigs.

Specificity of Twig Surface Extracts. In bioassays that use natural twigs, *Y. cagnagellus* shows an absolute oviposition preference for its host plant *E. europaeus* (Bremner et al., 1997). Our data show that females are able to choose the correct host only from the methanolic surface extract (Figure 6A and B). This implies that the compounds in the extract are mainly responsible for the choice of the host plant by the adult females. The stimuli are probably perceived by the insect with sensilla located on their antennae or tarsi (Hora and Roessingh, 1999). Nonhost extracts either do not contain these stimuli or possibly contain compounds deterring females from oviposition.

The compounds remain to be identified. Plant chemicals influencing oviposition in moths have been studied extensively, but only a few examples where specific compounds are identified are known (Honda, 1995). Moreover, even when active compounds are known, interpretation is complicated by fact that some Lepidoptera depend on synergistic action of various chemicals (Honda, 1990). Proportions of primary compounds (e.g., sugars or proteins) on the plant

surface can be species specific and may affect the reaction to the secondary compounds in the mixture (Soldaat et al., 1996).

Form and Surface Structure. Yponomeuta cagnagellus females seem to be well adapted to select the appropriate food plant for their offspring. One would expect the discrimination behavior of a specialist to reflect the suitability of the host plant for larval development (Wiklund, 1975). Specialist insects may be presumed to distinguish qualitative differences among host plants (Janz and Nylin, 1997). Thus, it is probable that various other cues, besides specific secondary compounds, will influence choice of the oviposition site. These may be chemical, visual, or tactile.

Although plant surface compounds seem to be a key stimulant, form and surface structure of the substrate also affect oviposition in *Y. cagnagellus*. The substrate needs to resemble the basic form of a twig (i.e., cylindrical), and flat filter paper is barely accepted, even if it is treated with twig surface extract. This seems adaptive behavior to distinguish leaves from twigs: leaves are an unsuitable oviposition site since they do not survive winter. This distinction is apparently not based on the surface compounds, as extracts of both leaves and twigs are acceptable for oviposition (Figure 4).

Surface texture affects the oviposition of many moths. They prefer smooth, hairy, or rough surfaces, depending on the peculiarities of their natural host plant(s) or relating to the species-dependent optimal condition for survival of the eggs (Ramaswamy, 1988; Renwick and Chew, 1994). The preference of *Y. cagnagellus* for paper tape (a rough surface) over glass (smooth) can be due to the rough surface of twigs in general. Alternatively, the tape-covered AT simply provides a better grip for the moths, making it more accessible.

CONCLUSION

Yponomeuta cagnagellus females appear to depend on a combination of specific chemical and tactile cues for host plant discrimination. If these are lacking, the moths will not oviposit. This is in contrast with the behavior of some (polyphagous) moth species that are reported to deposit eggs on blank controls and cage walls, even if generally better preferred substrates are available (e.g., Ramaswamy, 1988; Udayagiri and Mason, 1995). The fastidious discrimination behavior of *Y. cagnagellus* can be related to their specialization and tendency to deposit eggs in a few (1–10) large masses. This suggests that the moths are not time-limited in their search and discrimination behavior (Singer, 1984). It also implies there is strong selection pressure on females to be selective, as misplacement of an egg mass can lead to the loss of 10–100% offspring.

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RELATIONSHIP BETWEEN CHEMICAL FUNCTIONAL GROUPS ON *Eucalyptus* SECONDARY METABOLITES AND THEIR EFFECTIVENESS AS MARSUPIAL ANTIFEEDANTS

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Abstract—*Eucalyptus* displays strong intraspecific variation in resistance to browsing by marsupial folivores that can be attributed to variation in the concentration and type of diformylphloroglucinol compounds (DFPCs) in the foliage. In this study, we ask which functional groups of diformylphloroglucinol compounds determine their effectiveness in deterring feeding. We used a simple and highly deterrent compound, jensenone, as a model DFPC and compared its activity to structural variants that differ in the types of functional groups on the phloroglucinol molecule. Torquatone, a naturally occurring compound in the steam volatile fraction of *Eucalyptus torquata* foliage, has neither the aldehyde nor phenol groups that are believed to contribute to the antifeedant actions of jensenone. From the naturally occurring compounds we have synthesized two intermediates, a capped phenol/free aldehyde compound (acetyl-jensenone) and a free phenol/no aldehyde compound (demethyl-torquatone). Addition of jensenone and acetyl-jensenone to diets of common ringtail possums (*Pseudocheirus peregrinus*) substantially reduced their food intakes. Torquatone showed less activity, and there was little reduction in food intake when demethyl-torquatone was added to the diet. We conclude that at least the aldehyde groups attached to the aromatic nucleus are important in determining whether these compounds deter feeding by common ringtail possums, whereas the phenol groups may play only a minor role.

Key Words—*Eucalyptus*, *Pseudocheirus peregrinus*, jensenone, torquatone, plant secondary metabolite, feeding deterrent.

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INTRODUCTION

There have been many studies of the role of plant secondary metabolites (PSMs) in deterring feeding by mammalian herbivores. However, most have not quantified specific compounds but have used broad-scale assays of groups of compounds that share a similar functional group (such as phenolics (Oates et al., 1980; Cork, 1992; Kool, 1992; Hodar and Palo, 1997)) and/or react in a particular way with other dietary constituents (e.g., proteins and tannins (Provenza et al., 1990; Hume and Esson, 1993; McArthur and Sanson, 1993; Dearing, 1997)). Use of these measures has rarely resulted in clear-cut patterns because of differences in the activity of individual compounds that arise directly as a consequence of the particular molecular structure (Zucker, 1983; Waterman and Kool, 1994; Ayres et al., 1997). Increased understanding of the importance of PSMs in plant–herbivore interactions requires that we both identify and quantify, individually, the most active compounds.

Recent work on interactions between *Eucalyptus* and its marsupial folivores has shown that a newly discovered group of PSMs, the diformylphloroglucinol compounds (DFPCs), plays an important role in deterring feeding by marsupials (Lawler et al., 1998a,b; 1999a,b; Pass et al., 1998). Where our methods have progressed far enough to quantify individual compounds precisely [currently jensenone (Figure 1a) and sideroxylonals, dimers of jensenone (Figure 1e)], we have been able to show that in *Eucalyptus* species in which these are the predominant DFPCs they explain the majority of variation in feeding by marsupial folivores among individual trees (Lawler et al., 1999a; Lawler, unpublished data). However, in species where we cannot quantify all individual components, estimates of total DFPCs do not correlate nearly so well with feeding (Lawler et al., 1998a), suggesting that there is significant variation between different DFPCs in their deterrent activities.

A range of compounds based on acylated phloroglucinol with an isoprene or terpene side chain has been isolated from *Eucalyptus* foliage (Ghisalberti, 1996). All of the compounds thus far shown to be active against marsupial feeding have been DFPCs, characterized by two formyl (aldehyde —CHO) groups attached to the phloroglucinol molecule, but differing in the identity of and nature of bonding to the isoprene/terpene side chain (Figure 1a, e, f, g). Similarities in the levels of activity of macrocarpal G (Figure 1g), sideroxylonals, and jensenone (Lawler et al., 1998a,b, 1999a) and the much lower activity of torquatone (Figure 1b) have led us to suspect that it is the presence of aldehyde and/or phenol groups that determines and increases the deterrent activity of the DFPCs. The lower activity of euglobals (Pass et al., 1998) that have an ether linkage to the terpene side chain (Figure 1f), relative to macrocarpals that have free hydroxyls in both positions ortho to the terpene, also may indicate that the nature of the linkage between the terpene side chain and the phloroglucinol molecule and its effects on H-bonding are important.

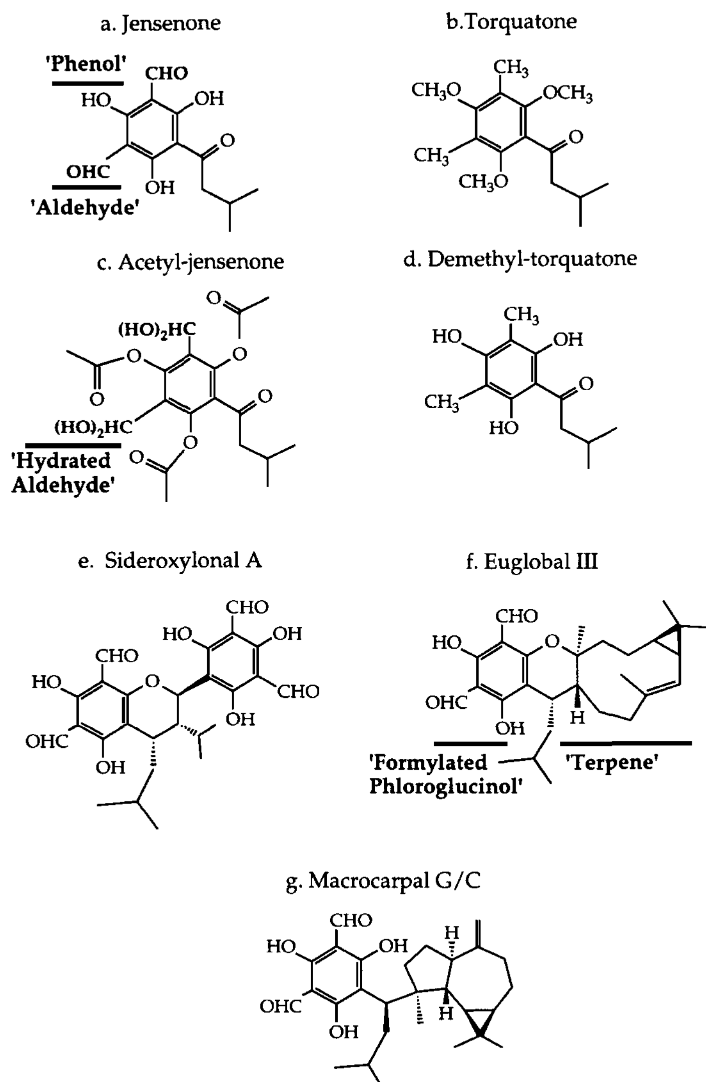

FIG. 1. Structures of compounds used in this study and related compounds known to act as antifeedants against folivorous marsupials.

In this study, we compared the effectiveness of four structurally similar and simple acylphloroglucinol derivatives in deterring feeding by common ringtail possums (*Pseudocheirus peregrinus*). Two of the compounds occur naturally in *Eucalyptus* foliage (jensenone and torquatone) (Figure 1a and b), whereas the

other two have been synthesized from these starting materials (Figure 1c and d). In these syntheses, we have removed or added functional groups with the intention of creating a series of compounds having the same basic structure and having both aldehyde and phenol groups (Figure 1a), neither aldehyde nor phenol groups (Figure 1b), phenol groups only (Figure 1d, demethyl-torquatone), and aldehyde groups only (Figure 1c, acetyl-jensenone). Note that the structure provided for acetyl-jensenone shows the $(HO)_2HC$ group where the aldehyde group is expected. This is a *gem*-diol, a group that is characterized by the ease with which it undergoes acidic cleavage (such as in an acid stomach) to revert to the aldehyde.

We conclude that the specific molecular structure is an important determinant of deterrent activity and that certain combinations of functional groups (e.g., aldehydes attached to aromatics) are fundamental to activity, but we do not yet have sufficient information on the metabolism of these compounds to identify the underlying mechanism that causes them to be such effective antifeedants.

METHODS AND MATERIALS

Animals. This research was approved by the Animal Experimentation Ethics Committee of the Australian National University and conforms to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Twelve common ringtail possums (*Pseudocheirus peregrinus*) were collected from *Eucalyptus* woodlands and kept individually in metabolism cages as described by Lawler et al. (1998a) and maintained on a basal diet made fresh each day. The diet consisted of (% wet matter) 55.5% grated apple, 28.3% banana pulp, 4.7% lucerne hay ground to pass a 2-mm sieve, 5.5% ground rice hulls, 4.7% ground Weetbix (a wheat-based breakfast cereal), and 1.6% acid casein. All animals maintained body mass on this basal diet.

The effectiveness of each compound as a feeding deterrent was tested by offering a diet treated with each of the compounds separately in varying concentrations in a no-choice protocol (see below for experimental design). Treated food was offered at 18 : 00 hr and removed at 06 : 00 hr the following morning, when all animals were given the untreated basal diet. On alternating nights, the basal diet alone was offered to prevent carryover effects between experimental nights.

Analysis. The four compounds tested were jensenone, torquatone, acetyl-jensenone, and demethyl-torquatone (Figure 1a–d). These represent a series of compounds with the same basic structure, but they vary in having phenol and aldehyde groups (jensenone), phenol (demethyl-torquatone), or aldehyde (acetyl-jensenone) groups, or neither phenol nor aldehyde groups (torquatone).

Jensenone and torquatone occur naturally in the foliage of *Eucalyptus*

jensenii and *E. torquata*, respectively (Bowyer and Jefferies, 1959; Boland et al., 1992), and they were extracted directly from leaves, while the two other compounds were each synthesized from one of the naturally occurring compounds.

Jensenone. Eucalyptus jensenii foliage, air-dried and ground to pass a 2-mm screen, was extracted in 20% acetone–light petroleum in a Soxhlet apprara-tus. The extracts were concentrated and combined with 1 liter of diethyl ether and washed three times with 0.3 M NaOH. These washes were acidified with hydrochloric acid (10 M), and the precipitate was washed with ethanol and recrystallized from acetone to give spectroscopically pure jensenone as a colorless solid (approximately 1.0%) (see Lawler et al., 1998b for details).

Torquatone. Torquatone was extracted from fresh *E. torquata* foliage by steam distillation with cohabation (Foley et al., 1987). The resulting oil was loaded onto silica and eluted with 10% ether–light petroleum. The pure torquatone-bearing fractions were identified by thin layer chromatography (TLC) and combined. The solvent was then removed in vacuo to yield torquatone as a low-melting-temperature solid. Spectroscopic data were as for the published method (Ghisalberti et al., 1995).

Acetyl-jensenone. To a stirred solution of jensenone (20.0 g, 0.075 mol) and pyridine (22.5 ml) in dichloromethane (dry, 200 ml) was added acetic anhydride (57 ml, 7 eq). The reaction was monitored by thin-layer chromatography and, when all the jensenone was consumed, 200 ml water were added. The water layer was removed and the organic layer washed with water (2 × 200 ml), sodium bicarbonate solution (2%, 2 × 200 ml), water (2 × 200 ml), hydrochloric acid (1.0 M, 2 × 150 ml) and water (2 × 200 ml). The organic layer was dried (sodium sulfate), the solvent removed, and the residue further dried under high vacuum to give jensenone triacetate (21.26 g, 66%) as a pale oily solid. Spectroscopic data closely corresponded with published data (Boland et al., 1992).

Demethyl-torquatone. A solution of torquatone (38.20 g, 0.136 mol) was stirred in concentrated sulfuric acid (120 ml) for 5 hr, after which TLC (10% methanol–dichlormethane) indicated that the reaction had gone to completion. The reaction mixture was poured onto water–ice (200 g), and the resultant slurry stirred in an ice bath. The mixture was adjusted to pH 12 by direct addition of KOH pellets (caution!), allowed to warm to room temperature, and washed with dichloromethane (3 × 300 ml). The aqueous layer was adjusted to pH 4 by the careful addition of sulfuric acid (50%, ca. 160 ml) and extracted with dichloromethane (3 × 400 ml), dried (magnesium sulfate), and the solvent removed in vacuo to yield demethyl-torquatone as a reddish oil (32.06 g, 99%). ^1H NMR ($CDCl_3$) δ 0.98 [6H, d, $—C(O)CH_2CH\underline{CH_3}$], 2.07 [2H, d, $—C(O)\underline{CH_2}CHCH_3$], 2.17 (1H, m, $—C(O)CH_2\underline{CH}CH_3$], 2.23 (3H, s, Ar—$\underline{CH3}$), 2.25 [3H, s, Ar$\underline{CH3}$]; MS (ES-MS) m/z 239 ($M+H^+$) and 261 ($M+Na^+$).

Experimental Design. To test whether the deterrence of each compound

was dose-dependent, five concentrations of each plus a solvent-treated control for each compound were offered to the 12 animals over eight nights in a balanced alpha-crossover design, giving four replicates for each treatment (John and Williams, 1995). All compounds were tested together to prevent confounding of time with treatments. The extra controls were used to statistically balance the design. Parameters of the model, with compound and concentration as fixed effects, were estimated by Restricted Maximum Likelihood (REML) theory (Cunningham, personal communication). Data were tested first for linearity across concentrations within each compound and, when found not to depart significantly from linearity ($\chi^2 = 15.3$, $df = 12$, $P = 0.225$), the slopes of the dose-dependent linear relationships were compared.

Concentrations of each compound offered were chosen primarily on the basis of preliminary data for deterrency of each of the naturally occurring compounds, as there was insufficient material of each of the synthesized compounds available for substantial preliminary testing. Jensenone is highly deterrent to feeding (Lawler et al., 1998b, 1999b) and so was offered at low concentrations, whereas torquatone is substantially less deterrent (see below, Lawler unpublished) and was added at higher concentrations. The concentrations of both these compounds in the artificial diet were similar to those found in *Eucalyptus* foliage. We assumed that the alteration in numbers of functional groups on the benzene ring would alter the activity of the synthesized compounds and thus varied the concentrations of the two synthesized compounds relative to their parent compounds. We expected that acetyl-jensenone would be less active than jensenone, and we used slightly higher concentrations than those for jensenone. We expected that the phenol groups on demethyl-torquatone would increase its activity relative to torquatone, and we used slightly lower concentrations. Concentrations of each compound are summarized in Table 1.

RESULTS AND DISCUSSION

Clearly, the particular chemical structure of a compound is important in determining its effectiveness as a deterrent to feeding by common ringtail possums. There were distinct differences in the activity of each of the four compounds (Figure 2, $\chi^2 = 32.97$, $df = 3$, $P < 0.001$). Jensenone was the most effective feeding deterrent, while acetyl-jensenone also was highly deterrent. Torquatone showed substantially less activity, and demethyl-torquatone the least. The compounds tested here differ only in the nature of the functional groups attached to the benzene ring, yet there is an order of magnitude difference in the amount of compound required to produce the same reduction in food intake.

Given such variation in activity among these structurally similar compounds, it is apparent that conventional "total" phenolic assays are not appropri-

TABLE 1. CONCENTRATIONS OF EACH COMPOUND USED IN NO-CHOICE BIOASSAY EXPERIMENT WITH CAPTIVE RINGTAIL POSSUMS

Jensenone			Acetyl-jensenone			Demethyl-torquatone			Torquatone		
	Concentration			Concentration			Concentration			Concentration	
Molecular weight	μmol/g dry matter	% wet matter	Molecular weight	μmol/g dry matter	% wet matter	Molecular weight	μmol/g dry matter	% wet matter	Molecular weight	μmol/g dry matter	% wet matter
266	7.52	0.06	428	13.63	0.18	238	70.03	0.50	280	119.04	1.00
	15.04	0.12		26.48	0.34		140.06	1.00		178.57	1.50
	30.08	0.24		50.62	0.65		210.08	1.50		297.62	2.50
	45.11	0.36		77.88	1.00		280.11	2.00		416.67	3.50
	60.15	0.48		101.25	1.30		350.14	2.50		535.71	4.50

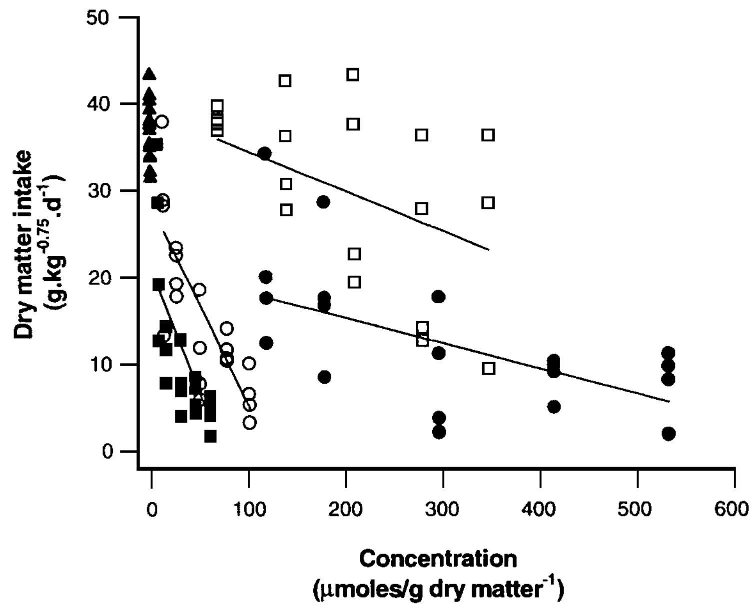

FIG. 2. Dose-dependent relationships between food intakes by common ringtail possums and the molar concentrations of potential antifeedant compounds. Solid squares are jensenone, open circles are acetyl-jensenone, solid circles are torquatone, open squares are demethyl-torquatone, and solid triangles are controls.

ate to assess the resistance of foliage samples to marsupial folivory. Such assay techniques (e.g., those based on Folin reagents) indicate only the presence of the particular functional group assayed and give no information on other functional groups found on the molecule. There is a wide variety of different phenolic compounds found in the foliage of eucalypts (Hillis, 1966). If differences as subtle as those among the compounds used in the experiments reported here can cause such powerful differences in activity, then assays that cannot discriminate between a tannin and a simple phenolic clearly do not have the resolution to explain variation in marsupial feeding among trees. For example, both jensenone and demethyl-torquatone contain the same number of phenolic groups per mole and, thus, would give the same response in a "total" phenolics assay, yet the difference in feeding activity between them is great. It should also be noted that jensenone and other DFPCs active against marsupial folivores are not extracted in conventional phenolic assays (Lawler et al., 1998a).

A range of similar compounds (including jensenone) has been investigated for other biological activity. Several macrocarpals exhibit antibacterial activity (Murata et al., 1990; Yamakoshi et al., 1992; Osawa et al., 1996), and siderox-

ylonal A inhibits attachment of mussel larvae (Singh et al., 1996). Grandinol, a simple acylphloroglucinol derivative (similar to jensenone but lacking one of the formyl groups) extracted initially from *E. grandis*, is a potent germination inhibitor, while similar compounds showed no such effect (Bolte et al., 1984). The euglobals (Figure 1f) have been investigated as potential antitumor agents, with some variation between euglobals of different structure. Detailed structure/activity studies have been carried out with a wide range of variations on the basic acylphloroglucinol structure (including DFPCs) to determine their importance in germination inhibition (Bolte et al., 1985) and inhibition of Epstein-Barr virus activation (as a de facto measure of antitumor activity) (Takasaki et al., 1990). These studies have been more comprehensive (i.e., covered a wider range of structural variation) than has been possible here because of the lower quantities of compound required for the bioassays. In both cases, they found substantial variation in activity associated with subtle changes in structure and were able to identify with reasonable precision the requirements for activity. Interestingly, the structural models for both germination and Epstein-Barr virus inhibition have similar requirements for the acylphloroglucinol structure with a formyl and a ketone group, which may apply also to deterrence of marsupial feeding. However, again, there are subtle differences in the requirements for activity. Jensenone was an effective Epstein-Barr virus inhibitor (Takasaki et al., 1990), but was less effective in inhibiting germination (Bolte et al., 1985; Boland et al., 1992).

A free phenolic group ortho to the isoprene unit was identified as important for the germination-inhibiting capacity of grandinol. That it may also be important in deterring feeding by marsupial folivores is suggested by the relative inactivity of torquatone. The reduced activity of euglobals, relative to macrocarpals (Pass et al., 1998), may indicate the need for free phenolic groups in both positions ortho to the isoprene/terpene group. Euglobals have an ether linkage at that position masking one of the phenolic groups, while this is lacking in the macrocarpals. Grandinol has not been tested against marsupials, but the groups of compounds shown to be active against marsupial feeding (macrocarpals, sideroxylonal, jensenone) all share this feature. Further support for this contention is provided by the close correspondence in the molar thresholds shown by common ringtail possums of jensenone and sideroxylonal. The molar threshold for jensenone in this experiment is similar to the molar threshold for sideroxylonal of Lawler et al. (1999a), although it should be noted that the threshold for jensenone might vary significantly between populations (Lawler et al., 1998b). Sideroxylonals are dimers of jensenone, and one might, therefore, expect the molar threshold for sideroxylonal to be half that of jensenone (while the threshold in milligrams would be similar). However, only one jensenone monomer has all three phenolic groups free, while the other has one bound in an ether linkage (similar to that found in euglobals), which may eliminate the deterrent effect of that part of the molecule.

The results obtained in this study were somewhat counter to those expected. As the synthesized compounds were intended to be intermediate in structure between the two naturally occurring compounds, intermediate activities were expected. This was the case with acetyl-jensenone, although both aldehyde and phenol groups were capped. However, as we noted, the *gem*-diol groups revert easily to aldehyde groups in acidic conditions. We suspected also that the high activity shown by this compound was due to its also losing the acetyl groups and reverting to the parent compound in the acid stomach conditions of common ringtail possums. Support for this hypothesis is given by the observation that this occurred while attempting to purify acetyl-jensenone after the acetylation reaction by column chromatography. Significant proportions of the resulting product were shown by TLC and NMR to be jensenone, while no jensenone was evident in the reaction end-product before chromatography (Eschler, unpublished). This result is most likely due to the acidic nature of the silica gel used in the column. However, only small amounts of jensenone were produced over several days when acetyl-jensenone was stirred in 0.1 M HCl (Eschler, unpublished). Our original intention was to produce a more appropriate intermediate by methylating jensenone to produce a compound with the aldehydes present and phenols absent, but our attempts to do so in sufficient quantity failed.

The lower activity of demethyl-torquatone relative to its parent compound may be a function of its increased polarity. It contains two of the features of the active compounds (phenol groups, ketone) and, therefore, may be expected to show greater activity than torquatone. However, the presence of the phenol groups also makes the demethyl-torquatone more polar and, thus, may reduce the likelihood of its being absorbed across lipid membranes. If the action of jensenone (and other DFPCs) is at the surface of the gut wall, then lipophilicity may be irrelevant, as may be indicated by the similar deterrency exhibited by jensenone, sideroxylonal, and macrocarpal G, which vary in polarity. There are currently few data on the fate of any DFPC after ingestion. We know that jensenone causes the release of serotonin, possibly from the gut wall, but cannot say whether this is a result of cell damage or stimulation of specific receptors (Lawler et al., 1998b). However, when jensenone is incubated in an isolated section of guinea pig gut, it disappears rapidly from the lumen, and debris accumulates, indicating cell damage. No jensenone or apparent metabolites can subsequently be found either in the lumen, the gut tissue, or the surrounding fluid. It appears that jensenone binds to proteins, but it is not yet known if this is specific to certain protein types, such as receptors in the gut wall (S. McLean and S. Brandon, personal communication). Thus, it is not possible at this stage to say whether absorption across the gut wall is necessary for these compounds to exert their effects.

Another feature of the DFPCs that may be important in determining the antiherbivore activity, and thus interpreting ecological importance, may be the

identity of the terpene moiety. In the only relevant study to date, Takasaki et al. (1990) compared the antitumor activity of a range of euglobals that differ only in the terpenoid part of the molecule. While there was some variation among compounds, all were active, and they concluded that the terpene part of the molecule was less important than the features of the acylphloroglucinol component described above. We suspect this also to be the case when considering deterrence of marsupial feeding, in light of the similarities in activity between jensenone, sideroxylonal, and macrocarpals. However, to date we have not been able to compare directly, for example, two macrocarpals with different terpene moieties, and we believe this is necessary before a firm conclusion is reached.

In conclusion, this structure–activity study has provided information that is useful in an ecological sense, illustrating the importance of identifying the specific molecular structures governing the plant–herbivore interaction. However, the quantities of each compound required for bioassays with whole animals, especially vertebrates, severely limit the number of compounds that can be synthesized in appropriate amounts, and this is perhaps reflected by the paucity of such studies in other plant–mammalian herbivore systems. In the only other study that we know of testing structural variants of a PSM against an herbivore that encounters it in the wild, only three compounds were tested (Clausen et al., 1986). In that study phenol groups were identified as being important for activity, but another cooccurring PSM with a phenol functionality was inactive. No attempt was made to understand the importance of other parts of the structure or the physiological effects of the compounds. Clearly, for plant–vertebrate herbivore systems, these limitations will, in most cases, prevent testing an extensive range of variations of the appropriate structure. This approach is unlikely, therefore, to lead to a complete understanding of the structural requirements. Detailed pharmacological studies of the metabolism of those compounds and the means by which animals can metabolize and tolerate or detoxify them may be a more profitable approach.

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USE OF ^{14}C-LABELED ALFALFA SAPONINS FOR MONITORING THEIR FATE IN SOIL

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Abstract—Alfalfa seedlings (cv. Cimmaron) were aseptically grown in a glass test tube containing a nutrient salts solution and ^{14}C-labeled sodium acetate into which filtered air was pumped. After five days of exposure to fluorescent light at 22°C, the alfalfa seedlings were removed, washed with cold water, and their saponins extracted. Mean yield of ^{14}C-labeled saponins was 50.2 mg/2.2 g of seed, and mean activity of the ^{14}C-labeled saponins was 5.1×10^4 dpm/mg. Thin-layer chromatography (TLC) of ^{14}C-saponins indicated soyasaponin I, medicagenic acid-3,28 glucoside, and medicagenic acid 3-*O*-glucoside, plus several unidentified spots, whereas TLC of hydrolyzed saponins (aglycones) showed medicagenic acid, hederagenin, and soyasapogenol B. After 150 hr at 22°C, 17.0% of the ^{14}C-labeled saponins added to a sterile clay loam soil were converted to $^{14}CO_2$, whereas 54.5% of the saponins were converted to $^{14}CO_2$ in the nonsterile soil. $^{14}CO_2$ evolution from each of four nonsterile soils that were amended with ^{14}C-labeled saponins was 57.4–69.9% after 14 days of incubation, and 2.4–24.0% of the added ^{14}C-labeled saponin was recovered in the humic acid fractions from the soils. Only 1.0–2.1% of the ^{14}C label was associated with microbial biomass, as estimated following chloroform fumigation of the soils. Use of ^{14}C-labeled saponins should facilitate a better understanding of the fate of these compounds in soil.

Key Words—Saponins, alfalfa, soils, humic acid, allelopathy.

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INTRODUCTION

Alfalfa saponins are known to exhibit allelopathic activity, reducing yields of succeeding plants following alfalfa cultivation (Guenzi et al., 1964; Golpen and Webster, 1969; Waller et al., 1995) and decreasing growth and/or activity of soil microorganisms (Levy et al., 1989; Oleszek et al., 1990b). The fate of these saponins in soil has received limited study to date. Inhibition of wheat seedling growth in soils treated with powdered alfalfa roots was less in heavy loam and loamy sand soils than in a loose or coarse sand (Oleszek and Jurzysta, 1987). In this work, alfalfa root saponins were adsorbed from water solutions more by heavy-textured soils than by light-textured soils. Bioassay with *Trichoderma viride* of inhibitory saponins left in water after incubation with soil was used to estimate saponin adsorption. Bioassays, however, are an indirect method for following the fate of saponins in soil.

Solvent extraction from soil followed by cleanup and HPLC (Oleszek et al., 1990a) quantification of saponins is a more direct method of monitoring the fate of saponins over time; however, repetitive destructive sampling and complicated chemical analysis may be problematic. Use of ^{14}C-labeled saponins offers an alternative approach to following saponin fate in soils. ^{14}C-labeled saponins have been used to understand saponin synthesis and distribution in planta (Nowacki et al., 1976; Fuggersberger-Heniz and Franz, 1984) but have yet to be used to shed light on the partitioning of these compounds in soils and soil fractions.

In this paper, we describe the production of ^{14}C-labeled alfalfa saponins and their use in studying the fate of saponins in four soils that differ in physicochemical characteristics.

METHODS AND MATERIALS

Production of ^{14}C-Labeled Alfalfa Saponins. ^{14}C-labeled alfalfa seedlings were prepared by using the procedure of Nowacki et al. (1976) with modifications. Seeds (2.2 g) of alfalfa (*Medicago sativa* L. cv. Cimmaron) were stirred in 20 ml of concentrated H_2SO_4 for 20 min and then aseptically rinsed in five changes of sterile water (20 ml) inside a laminar flow hood. This procedure completely rid the seeds of microorganisms, as determined by the absence of microbial colonies from treated seeds plated on dishes of dilute trypticase soy broth agar (TSA) (Martin, 1975b). Germination of treated seed was 83%. Seeds were placed in a sterile 28 cm × 1.6 cm diam. glass test tube containing 2 ml of a filter-sterilized solution of 20 μCi/ml ^{14}C-labeled sodium acetate (1,2-^{14}C; 4.29 GB/mmol; ICN, Irvine, California) in 48 ml Hoagland's solution (Hoagland and Arnon, 1950). The solution pH was 5.5. The tube was aseptically sealed with a rubber stopper fitted with glass tubes for the passage of filtered air (Gelman;

0.2 μm pore diam.) at 30 cc/min through the solution. After five days of incubation at 22°C, under continuous fluorescent light inside a laminar flow hood, the seedlings were transferred to filter paper on a Buchner funnel under vacuum and washed three times with ca. 30 ml of ice-cold water to remove external ^{14}C. Fresh weight of seedlings was 20–26 g. Seedlings were cut into 3- to 4-mm pieces, placed in a paper thimble that was inserted in a Soxhlet extractor, and extracted in 250 ml of methylene chloride for 16 hr at 80°C. The methylene chloride was discarded, and the tissue was extracted with 200 ml of 80% methanol for 4 hr at 50°C. The methanol solution was filtered, concentrated at 50°C under vacuum to near-dryness, and redissolved in 15 ml of 30% methanol. A C-18 PrepSep column (Fisher Scientific, Fair Lawn, New Jersey) was preconditioned with 5 ml of 100% methanol, followed by 30% methanol. The 30% methanol solution containing saponins was applied to the column, followed by 5 ml of 30% methanol, and saponins were eluted with 5 ml of 100% methanol. This eluted fraction was concentrated at 50°C under vacuum to near dryness and further dried under a stream of dry nitrogen. The recovered saponins were measured for their ^{14}C activity. One-milligram samples in each of three vials containing 10 ml of Ecolume (ICN) cocktail were counted for 5 min in a Beckman LS 5000C5 liquid scintillation counter (LSC) (Beckman, Instruments, Fullerton, California).

Saponins in the ^{14}C-labeled saponin fraction were identified by thin-layer chromatography (TLC) by comparing the elution of spots in the ^{14}C-labeled saponin fraction before and after acid hydrolysis with standards of saponins and aglycones obtained from G. Waller and W. Oleszek. The purity of these standards had been determined by GC-MS (Oleszek et al., 1990b; Waller et al., 1995). Saponins were analyzed on Silica Gel 60 developed in ethyl acetate–acetic acid–water (7 : 2 : 2 v/v), and aglycones were analyzed on Silica Gel RP-18 F245 plates developed in petroleum ether–chloroform–acetic acid (7 : 2 : 2 v/v). Spots were visualized by spraying the plates with Lieberman-Burkhard reagent, heating at 120°C, and observing the plates under natural and ultraviolet light (Oleszek and Jurzysta, 1987).

Soils. Port silt loam and Norge clay loam soils were obtained from agronomy fields surrounding Oklahoma State University, Stillwater, Oklahoma, USA, and low-humic Andsol silt loam and Gray Upland clay loam soils were collected from fields of the Hokkaido Toachi Agricultural Experiment Station, Hokkaido, Japan. Soils were sieved to pass a 2.8-mm sieve, and stored in plastic containers with lids at 4°C. Characteristics of the soils, excluding percent humic acid composition, were determined by the Soil Fertility Laboratory of Oklahoma State University and are shown in Table 1. Percent humic acid was estimated according to Schnitzer (1982). Soils were equilibrated overnight to room temperature (22°C) before use.

Soil Respiration of [^{14}C] Saponins. The ability of the microflora of soils

TABLE 1. PHYSICOCHEMICAL CHARACTERISTICS OF SOILS

Soil	Bulk density (g/cc)	pH	CEC (cmol(+)/kg)	Humic acid (mg/g)	Percent				
					Sand	Silt	Clay	Total C	Total N
Port silt loam	1.1	7.1	3.0	1	78.5	10.4	11.1	0.7	0.07
Norge clay loam	1.0	6.3	6.0	1	58.2	18.8	23.0	0.6	0.06
Andsol silt loam	0.8	6.0	15.5	8	67.1	28.0	4.9	2.5	0.25
Gray Upland clay loam	0.9	5.5	30.1	22	21.6	47.4	30.0	5.7	0.39

to utilize [^{14}C] saponins was determined by measuring $^{14}CO_2$ evolved by soil respiration. The apparatus for measuring soil respiration consisted of glass jars (4.3 cm diam. × 4.5 cm) that were sealed with rubber stoppers fitted with glass tubes for the passage of moist, filter-sterilized (0.2 μm pore) air through the jar (Filonow et al., 1996). Soils in jars were amended with [^{14}C] saponins, the jars were sealed, incubated at 22°C, and $^{14}CO_2$ evolved was periodically trapped in 10 ml of Ecolume cocktail containing 1 ml of Hyamine hydroxide (ICN) and counted.

Respiration of [^{14}C] saponins in a nonsterile soil was compared to a sterile soil. Two grams of a Norge clay loam at 25% water-holding capacity was placed in each of four jars. Two of the jars were autoclaved (135°C; 15 psi) for 1 hr, and the others were not autoclaved. Water containing 2×10^4 dpm of ^{14}C-labeled saponins per milliliter was filter-sterilized (0.2 μm pore diam.), and 1 ml of saponin solution dispensed into each jar. $^{14}CO_2$ was collected after 1, 5, 12, 24, 48, 96, 120, and 150 hr. After every 24 hr, each jar was opened briefly, 200 μl of sterile water were added to the soil to maintain soil moisture, and the jar sealed again. At termination of the experiments, soils were frozen for later humic acid extraction.

Respiration of ^{14}C-labeled saponins in four nonsterile soils was compared. Two grams each of Port silt loam, Norge clay loam, Andsol silt loam, and Gray Upland clay loam soils were added to the jars, treated with 1 ml of ^{14}C-labeled saponins (1.8×10^4 dpm/ml), and the $^{14}CO_2$ evolved measured every 24 hr for 14 days. After every 24 hr the jars were briefly opened, and 400 μl of sterile water was added to each soil. There were three replicates per soil. Soils were later frozen and saved for humic acid extraction.

In another experiment, 2.5 g quantities of soils were placed into jars, and the same protocol for amending the soils and measuring $^{14}CO_2$ was used as previously described. At the end of $^{14}CO_2$ collection, however, the soils were saved for the determination of ^{14}C in the microbial biomass of the soils. There were three replicates per soil, and one experiment was conducted.

[^{14}C] Humic Acid Extraction. At the end of the above experiments, the humic acid of the soils was extracted and the ^{14}C in the humic acid determined. Extraction of humic acid (Schnitzer, 1982) in the four soils was scaled down to assay small quantities. Two grams of soil were transferred to a 50-ml polypropylene centrifuge tube containing 10 ml of 0.5 N NaOH. The tube was capped and shaken at 22°C for 16–18 hr. The NaOH extractant and soil were centrifuged at 1100 *g* for 30 min and the supernatant decanted and saved at 4°C. Another NaOH extraction of the soil was done for 6 hr, centrifuged, and the supernatants combined. The combined supernatant from each soil was adjusted to pH 1 with 5 N HCl, and centrifuged at 3250 *g* for 30 min. The precipitate (humic acid) was recovered, washed three times with water, dried at 105°C, and the ^{14}C in each humic acid sample determined.

^{14}C in Microbial Biomass of Soils. ^{14}C in the microbial biomass of the four soils was determined with the chloroform fumigation method (Vance et al., 1987). At the end of a 2.5-g soil respiration experiment, 1.0 g of each soil was placed in a 250-cc screw-capped glass jar containing 5 ml of chloroform. The jars were sealed, and the soils fumigated for 24 hr at 25°C. Soils were removed, shake-extracted with 20 ml of 0.5 M K_2SO_4 (Vance et al., 1987) in a sealed 50 ml centrifuge tube for 30 min, and centrifuged for 10 min at 1100*g*. One gram each of nonfumigated soils also was extracted for comparison. ^{14}C in 5 ml of each supernatant in 15 ml of Econolume cocktail was determined by LSC. ^{14}C activity in the extractable carbon from fumigated soil minus that in the extractable carbon from nonfumigated soil was used as an estimate of ^{14}C activity in the microbial biomass of the soils.

Data Analysis. Experiments were repeated once, except where otherwise noted in the text. Data for ^{14}C in humic acid or in microbial biomass were subjected to an analysis of variance and means separated by LSD test ($P = 0.05$). Data for $^{14}CO_2$ evolved from soils were presented as the mean ± one standard deviation (SD).

RESULTS AND DISCUSSION

Production of [^{14}C] Saponins. The mean dry weight yield of saponins was 50.25 mg for 2.2 g of seed, or 4.54% of the dry weight of the seedlings in three experiments. The mean ^{14}C activity per milligram of dry saponin was 5.1×10^4 dpm, which was nearly twice that found by Nowacki et al. (1976), who reported 27,370 dpm/mg of dry saponin after six days of radiolabeling. Thin-layer chromatography of our [^{14}C] saponins showed the presence of several compounds reported by others (Oleszek et al., 1990b; Wyman-Simpson et al., 1991; Waller et al., 1995), including soyasaponin I, medicagenic acid 3-28 glucoside, and medicagenic acid 3-*O*-glucoside. Several spots were unidentified. TLC of hydrolyzed

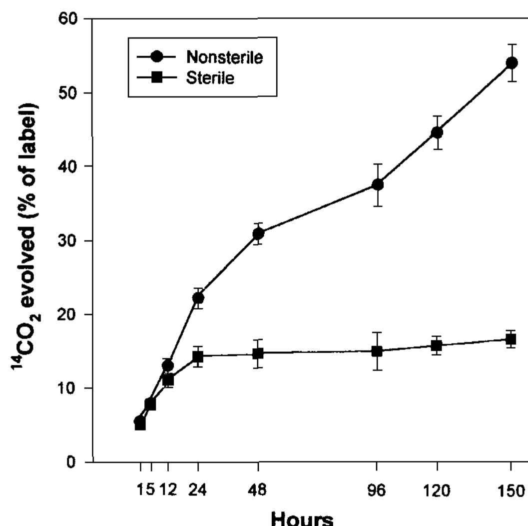

FIG. 1. $^{14}CO_2$ evolved from a sterile and nonsterile Norge clay loam soil amended with ^{14}C-labeled saponins. Values are the means of two replicates ± SD.

saponins (aglycones) showed medicagenic acid, hederagenin, and soyasapogenol B. We did not quantitate the recovery of individual saponins or aglycones. However, Nowacki et al. (1976), who used [^{14}C] acetate, and Peri et al. (1979) who used [^{14}C] mevalonate for labeling alfalfa reported that after 6–10 days of incubation, 63–64% of the total ^{14}C activity had been incorporated into medicagenic acid.

There are other approaches to ^{14}C-labeling of plants, such as [^{14}C] melavonate applied to germinating seeds, or [^{14}C] squalene applied to leaves (Peri et al., 1979). $^{14}CO_2$ has also been applied to plants in air-controlled growth chambers (Martin, 1975a). However, we found our modified labeling procedure with [^{14}C] acetate applied to germinating alfalfa seeds easy to do, and it yielded an acceptable specific activity (ca. 5×10^4 dpm/mg). It may be possible to improve the labeling procedure to increase the specific activity; an activity of 10-fold greater would be desirable.

Soil Respiration of [^{14}C] Saponins. Timed measurement of $^{14}CO_2$ evolved from amended soils was simple and direct. Alfalfa saponins were rapidly degraded in nonsterile soil compared to sterile soil. Microbial degradation was obvious in the nonsterile Norge clay loam soil, in which 50% of the [^{14}C] saponin label had been converted into $^{14}CO_2$ after five days of incubation (Figure 1). $^{14}CO_2$ output from nonsterile soil continued to increase throughout the experiment, and after 150 hr, it was 54.5% of the label. Sterile soil also produced

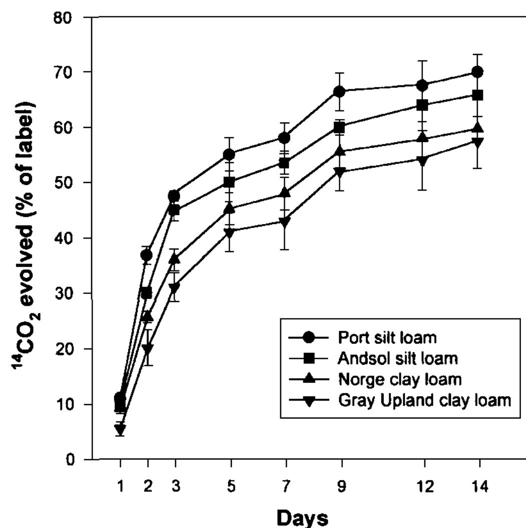

FIG. 2. $^{14}CO_2$ evolved from four nonsterile soils amended with ^{14}C-labeled saponins. Values are the means of three replicates ± SD.

$^{14}CO_2$ within 5 hr of pulsing the soil, but thereafter showed little change; it was 17.0% of the label after 150 hr. Sterile soil sprinkled on dilute TSA plates at the end of the experiment produced no microbial colonies after seven days of incubation at 22°C, suggesting that briefly opening the jars to water the soils did not introduce microbes that might degrade [^{14}C] saponins. However, degradation of [^{14}C] saponins by microbes that are not easily recovered by plate-culture methods (Martin, 1975b) cannot be ruled out.

Another explanation for the apparent respiration of [^{14}C] saponins in sterile soil may be nonbiological degradation due to saponin adsorption to humic acid. The ^{14}C in humic acid recovered from the sterile soil (30.9% of the [^{14}C] saponin label) was sevenfold greater than the ^{14}C in humic acid recovered from the nonsterile soil (4.3%). Humic acids are known to adsorb pesticides and catalyze their structure (Stevenson, 1994). Conceivably, humic acid also may affect a nonbiological alteration of saponins, resulting in CO_2 release.

$^{14}CO_2$ evolution from four nonsterile soils augmented with [^{14}C] saponins showed a sigmoidal pattern characteristic of microbial growth (Figure 2). The soils did not differ in their respiration, except for the Port silt loam and the Gray Upland clay loam, which differed at some sampling times but not at the end of the experiment. After 14 days of incubation, $^{14}CO_2$ evolution as a percent of the label from the Port silt loam, Norge clay loam soil, Andsol silt loam, and Gray Upland clay loam was 69.9%, 59.7%, 65.7%, and 54.7%, respectively.

TABLE 2. PERCENT $^{14}CO_2$ EVOLUTION FROM SOILS AND ^{14}C DISTRIBUTION IN SOIL FRACTIONS AFTER 14 DAYS OF INCUBATION WITH ^{14}C-LABELED SAPONINS[a]

Soils	Percent ^{14}C in		
	CO_2	Humic acid	Biomass
Port silt loam	69.9a	2.4a	2.1a
Norge clay loam	59.7a	2.4a	2.0a
Andsol silt loam	65.7a	14.8b	1.0a
Gray Upland clay loam	54.7a	24.0c	2.1a

[a]Values in a column followed by the same letter were not significantly different ($P = 0.05$) from each other according to the LSD test.

^{14}C was found in the humic acid fractions from all soils (Table 2), suggesting [^{14}C] saponins from alfalfa were adsorbed by humic acid or became structurally incorporated in humic acid. To our knowledge, this is a new finding. Humic acid from each of the Oklahoma soils was 2.4% of the label, whereas ^{14}C in humic acids from the Gray Upland clay loam and Andsol silt loam (Japanese soils) was 24.0% and 14.8%, respectively. The greater percent of ^{14}C in humic acid from the Japanese soils compared to the Oklahoma soils probably reflects the increased adsorptive capacity of the Japanese humic acid fractions. Additional studies should be conducted to investigate the interaction of humic acid and saponins in soil, and the role such interactions play in regulating allelopathic potential of soils.

Soils did not differ in the capacity of their microbial biomass to sequester [^{14}C] saponins (Table 2). Only 1.0–2.1% of the label was associated with microbial biomass. This represents the ^{14}C that was in cytoplasm released when microbial cells were lyzed by chloroform; however, the ^{14}C adsorbed to exterior surfaces of microbial cells cannot be estimated by the methods used in the present study, so our recoveries were most likely an underestimate of the saponins taken up by microbial biomass. Nonetheless, use of [^{14}C] saponins allowed us to estimate the amount of alfalfa saponins that partitioned into the microbial biomass.

In summary, we describe herein the first use of [^{14}C] saponins for monitoring the fate of saponins from alfalfa in soils or soil fractions. Additional studies with [^{14}C] saponin should facilitate a better understanding of the fate of these compounds in soils from different cropping systems, and aid the study of allelopathy.

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EVALUATION OF FERULIC ACID UPTAKE AS A MEASUREMENT OF ALLELOCHEMICAL DOSE: EFFECTIVE CONCENTRATION

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Abstract—Soil solution concentrations of allelopathic agents (e.g., phenolic acids) estimated by soil extractions differ with extraction procedure and the activities of the various soil sinks (e.g., microbes, clays, organic matter). This led to the hypothesis that root uptake of phenolic acids is a better estimator of dose than soil solution concentrations based on soil extracts. This hypothesis was tested by determining the inhibition of net phosphorus uptake of cucumber seedlings treated for 5 hr with ferulic acid in whole-root and split-root nutrient culture systems. Experiments were conducted with 11 ferulic acid concentrations ranging from 0 to 1 mM, phosphorus concentrations of 0.25, 0.5, or 1 mM, and solution pH values of 4.5, 5.5, or 6.5 applied when cucumber seedlings were 9, 12, or 15 days old. The uptake or initial solution concentration of ferulic acid was regressed on ferulic acid inhibition of net phosphorus uptake. Attempts were made to design experiments that would break the collinearity between ferulic acid uptake and phosphorus uptake. The original hypothesis was rejected because the initial ferulic acid solution concentrations surrounding seedling roots were more frequently and consistently related to the inhibition of net phosphorus uptake than to ferulic acid uptake by these roots. The data suggest that root contact, not uptake, is responsible for the inhibitory activity of phenolic acids.

Key Words—Allelopathy, dose, ferulic acid, phosphorus uptake, *Cucumis sativus*, cucumber.

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INTRODUCTION

Understanding how inhibitory allelopathic agents affect plant physiological processes, such as hydraulic conductivity or nutrient uptake by roots and ultimately growth, requires determination of the effective concentration or dose required for a detectable level of inhibition. Soil-solution concentrations of allelochemical agents that may correlate with inhibitory events are not necessarily representative of root dose since soils contain a number of sinks (e.g., microbes, clays, organic matter) besides roots for these allelopathic agents. Soil-solution concentrations, which are invariably estimated by soil extractions, differ with extraction procedure (Dalton et al., 1987; Blum et al., 1992, 1994; Blum, 1997) and the activities of the various soil sinks. This has led researchers to hypothesize, assume, or imply that root uptake (micromoles per gram root per unit time) of allelopathic agents is a better estimator of dose than soil solution concentrations based on soil extracts (Willis, 1985; Shann and Blum, 1987; Lyu and Blum, 1990). To test this hypothesis, the uptake and initial solution concentration of ferulic acid, a commonly identified allelopathic agent (Rice, 1984), were individually regressed on ferulic acid inhibition of net phosphorus uptake by intact cucumber seedlings in nutrient culture. Attempts were made to design experiments that would break the collinearity between ferulic acid uptake and phosphorus uptake. Ferulic acid is commonly found in plants (Bates-Smith, 1956; Goodwin and Mercer, 1983; Siqueira et al., 1991; Harborne, 1993), leaf leachates (Abdul-Rahman and Habib, 1989), root exudates (Tang and Young, 1982; Tang, 1986), plant debris (Chou and Patrick, 1976; Whitehead et al., 1983; Kuiters and Sarink, 1986; Kuiters, 1990; Blum et al., 1991), and soils (Whitehead, 1964; Guenzi and McCalla, 1966; Shindo et al., 1978; Whitehead et al., 1982, 1983; Kuiters and Denneman, 1987; Blum et al., 1991). Cucumber (*Cucumis sativus* cv. Early Green Cluster) was chosen as the bioassay species because the inhibitory effects of ferulic acid on cucumber water utilization, net nutrient uptake, and growth have been characterized in considerable detail (Blum and Dalton, 1985; Blum and Rebbeck, 1989; Booker et al., 1992). Nutrient culture was used to control solution concentrations surrounding roots and to determine ferulic acid uptake by roots in the absence of other soil sinks (e.g., clay, organic matter, soil microbes).

METHODS AND MATERIALS

General Aspects. Cucumber seeds (*Cucumis sativus* cv. Early Green Cluster) were germinated in trays of vermiculite in the dark at 30°C for two days. The seedlings were then exposed to 11 hr of light and 12 hr of dark before transplanting them into jars containing 110 ml of Hoagland's solution (pH 5.5) (Hoagland and Arnon, 1950). The seedlings were suspended by foam collars

inserted through holes in the lids of the jars. The jars were wrapped in aluminum foil to exclude light from the root zone. Prior to treatment, seedlings were grown for 12 days (unless otherwise noted) in a growth chamber in the Southeastern Plant Environmental Laboratory under a PPFD of 400 μmol/m/s (supplied by fluorescent and incandescent lights), a 12 hr photoperiod, and 26/22°C day/night temperatures (NCSU Phytotron; Downs and Thomas, 1991). Deionized water was added daily to replace water lost via evapotranspiration. The nutrient solutions were changed four days after the seedlings were transplanted. Nutrient solutions were replaced with 0.5 mM $CaSO_4$ solutions one day before experimental treatments began. Seedlings were treated in a system of small plastic beakers (50 ml) enclosed within a larger square container. Roots were suspended through a hole in the lid of the large container and placed into 40 ml of treatment solution(s) (pH 5.5, unless otherwise noted) in the 50-ml beakers. The large container was wrapped in foil to exclude light from the system. In addition to the range of ferulic acid concentrations, all treatment solutions contained 0.5 mM $CaSO_4$, 5 mM 2(*N*-morpholino) ethanesulfonic acid (MES buffer), and 0.5 mM KH_2PO_4 unless otherwise stated. Preliminary experiments indicated that 5 mM MES buffer had no detectable effect on net phosphorus or water uptake and that the buffer adequately stabilized the pH at 5.5 during the time frame of an experiment (5 hr). The net uptake of ferulic acid (FER), water, and phosphorus (Pi) was based on depletion of these substances from solution. The FER remaining after 5 hr of treatment was determined by the Folin and Ciocalteau (FC) method (Box, 1983), which was determined by preliminary experiments to be highly related to HPLC determinations of FER [HPLC concentration = −9.39 + 0.89 (FC concentration); $P = < 0.0001$; $R^2 = 0.98$]. The Pi remaining at the end of the experiments was determined by a spectrophotometric method (Taussky and Shorr, 1953). The water remaining was measured to the nearest 0.5 ml in a graduated cylinder and was used in calculations of the amount of FER and Pi remaining in solution at the end of the experiment. Preliminary experiments indicated that an aerated system utilizing 40 ml of solution per cup and a 5-hr treatment period provided adequate Pi uptake for detection and did not limit Pi availability by the end of the time period. Previous studies by Shann and Blum (1987) indicated that there was minimal microbial utilization of FER over the 5 hr and that uptake measured as depletion from solution was closely related to isotope net uptake over the same time period. All uptake values were expressed on a per dry root weight basis since preliminary experiments indicated that root dry weight was closely related to root fresh weight [mg root dry wt = −0.06 + 0.02 (mg root fresh wt); $P < 0.0001$; $R^2 = 0.94$], root surface area [mg root dry wt = 3.13 + 1.55 (root surface area); $P < 0.0001$; $R^2 = 0.90$], and root volume [mg root dry wt = −1.35 + 20.00 (root volume); $P < 0.0001$; $R^2 = 0.87$]. Fresh weights of roots blotted dry were determined gravimetrically immediately after harvest. Dry weights were determined gravimetrically after oven drying at

40°C for 48 hr. Root surface area was estimated by direct readings from a leaf area meter. Root volume was measured by the displacement of water in a closed volumeter tube system modeled after Novoselov (1960).

Experiments designed to determine how various factors influence ferulic acid uptake and/or ferulic acid inhibition of net phosphorus uptake were as follows:

Ferulic Acid Concentration. Seedling roots were treated in solutions containing 11 FER concentrations ranging from 0 to 1.0 mM. Actual FER concentrations used for regressions were based on FC analysis of the originally prepared solutions. There were five replicates for a total of 55 seedlings.

Phosphorus Concentration. Since the linear portion of the net FER uptake curve in a previous experiment fell between 0.1 and 0.7 mM FER, this FER concentration range was used for subsequent experiments. In this experiment seedlings were treated with four FER concentrations (0, 0.25, 0.5, or 0.75 mM) and three Pi concentrations (0.25, 0.5, or 1.0 mM), added as KH_2PO_4. There were four replicates for a total of 48 seedlings. All other experiments contained 0.5 mM Pi.

Seedling Age. Previous studies by Shann and Blum (1987) suggested that FER uptake was modified by seedling age and/or size. In this experiment, 9-, 12-, or 15-day-old seedlings were treated with five FER concentrations (0, 0.2, 0.4, 0.6, or 0.8 mM). There were three replicates in this experiment for a total of 45 seedlings. All other experiments, including the two previous experiments, utilized 12-day-old seedlings.

pH. FER uptake has previously been reported to be influenced by soil-solution pH (Shann and Blum, 1987). Seedlings were treated with four FER concentrations (0, 0.25, 0.5, or 0.75 mM) at pH 4.5, 5.5, or 6.5. The pH of solutions was adjusted with NaOH or HCl, and 5 mM MES buffer was added to stabilize solution pH throughout the experiment. There were five replicates in this experiment, for a total of 60 seedlings. The solution pH used in all other experiments was 5.5.

Justification for Using MES Buffer. Over the pH values tested neither phosphate nor the zwitterionic buffer MES exist in an uncharged form that could freely permeate membranes. Anion uptake through the plasma membrane of plants requires symport of a cation (in most cases protons) to overcome the inside negative membrane potential. Channel carrier proteins have highly specific substrate binding sites that make it very unlikely that structurally different inorganic and organic anions, such as phosphate and ferulic acid, or zwitter ions, such as MES, compete for the same binding sites. This is supported by the finding that ferulic acid uptake was not affected by external phosphate concentrations. MES is frequently used as an inert buffer in ion transport studies and, to our knowledge, does not interfere with ion uptake in the concentrations used in this study.

Container Size. Seedlings were treated with four FER concentrations (0,

0.25, 0.5, or 0.75 mM) in four sizes of plastic containers (20, 40, 60, or 80 ml of treatment solution). The standard container size used for all other experiments was 40 ml. All container sizes in this experiment were constructed to have virtually identical diameters and the same amount of the root system was exposed to solution in all cases (i.e., whole root system, except approximately 1.5 cm at the top, which was suspended in the air gap between the suspension collar and the top of the solution). Thus, the only differences between the container sizes were the height of the container and the total number of FER molecules in solution. The smallest container sizes were elevated to keep the light intensity at the plant level the same for all treatments. There were four replicates in this experiment, for a total of 64 seedlings.

Split-Root Experiment. Portions of the root system (approximately 25. 50, 75, or 100%) were exposed to treatment solutions containing 0.25, 0.5, or 0.75 mM FER. The remainder of the root system was placed in a second container with 0 mM FER treatment solution. There were four replicates of each proportion—FER concentration combination, plus four control plants which had their entire root system in 0 mM FER. The total number of plants was 52.

Data Analysis. A completely randomized block design was used for all experiments. Because the effect of blocking was insignificant for all experiments, blocks were not included as a factor in the final analyses. Control means for Pi uptake per gram of root dry weight were determined for each experimental condition (e.g., pH, age) and used to calculate percent inhibition of Pi uptake as follows:

$$[(\text{control mean Pi uptake/g root} - \text{Pi uptake/g root of treatment})/\text{control mean Pi uptake/g root}] \times 100.$$

Data were subjected to analysis of variance and subsequent regression analyses by using the JMP statistical package (SAS Institute, Inc., 1994). Polynomials were used to smooth the data to provide the reader with general patterns or trends. No attempt was made to determine the exact curves or to account for all the potential variability in the data. Statistical significance was based on $\alpha \leq 0.05$. The split-root experiment was analyzed with a variable created by multiplying the concentration of FER by the proportion of roots exposed to FER (Lehman et al., 1994). The proportion of roots exposed was based on the actual value derived from dry weights of the roots.

RESULTS

Ferulic Acid Concentration. A curvilinear relationship was found between ferulic acid (FER) concentration and net FER uptake (milligrams per gram of

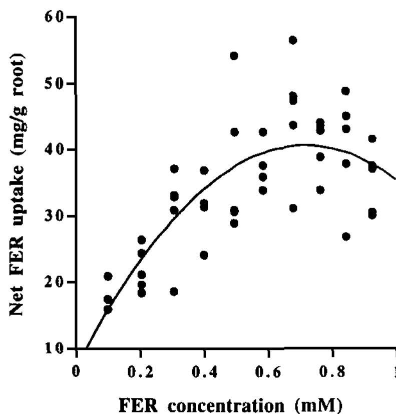

FIG. 1. Net uptake of ferulic acid (FER) by 12-day-old cucumber seedlings treated in nutrient solutions (pH 5.5) containing 0.1–1.0 mM FER. Equation of regression model: mg FER uptake/g root dry wt = 2.99 + 111.35 (FER concentration) – 80.64 (FER concentration)^2; $P < 0.0001$; $R^2 = 0.78$.

root depletion from solution) over the range tested (0–1.0 mM); as FER concentration was increased, FER uptake increased and then leveled off (Figure 1). The linear portion of the curve was estimated to be between 0.1 and 0.7 mM FER by a stepwise procedure (highest concentration data were removed individually until the best-fit linear regression was obtained). This FER concentration range was then used in subsequent experiments. The percent inhibition of net phosphorus (Pi) uptake (based on depletion from solution) increased and then leveled off as the FER concentration was increased and as the FER uptake per gram of root increased. The regression model for percent inhibition of net Pi uptake by FER concentration had an R^2 of 0.77 compared to an R^2 of 0.39 for the regression model for percent inhibition of net Pi uptake by FER uptake/g root. Both regression models were quadratic (Figures 2 and 3). The linear portion of the percent inhibition Pi uptake by FER concentration curve was estimated by a stepwise procedure to be between 0.1 and 0.8 mM FER.

Phosphorus Concentration. Net FER uptake was not affected by the Pi concentration in the solution over the range of concentrations tested (0.25–1.0 mM Pi; e.g., Table 1). The relationship between net FER uptake and FER concentration (mM) was similar to that observed in the previous experiment. The regression model was as follows: net FER uptake/g root dry wt = 0.44 + 144.01 (FER conc) – 129.46 (FER conc)^2; $P < 0.0001$; $R^2 = 0.92$.

For the control group (0 mM FER), net Pi uptake per gram of root dry wt was not affected by the Pi concentration over the range of concentrations tested. The net Pi uptake of controls was 3.78 ± 0.50 mg/g root dry wt (mean ± standard

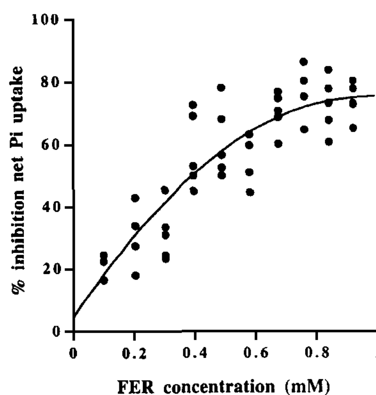

FIG. 2. Inhibition (% reduction from controls) of net phosphorus (Pi) uptake in 12-day-old cucumber seedlings treated in nutrient solutions (pH 5.5; 0.5 mM Pi) containing 0.1–1.0 mM ferulic acid (FER). Equation of regression model: % inhibition net Pi uptake = 3.67 + 144.16 (FER concentration) − 73.30 (FER concentration)^2; $P = 0.0050$; $R^2 = 0.77$.

error). The effect of FER concentration on percent inhibition of net Pi uptake was modified by the Pi concentration in the solution (significant interaction; $P = 0.0249$) such that the inhibitory effects of FER decreased as the Pi concentration was increased. The slopes of 0.25, 0.5, and 1.0 mM Pi regressions were all

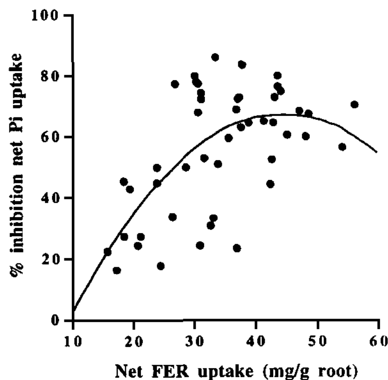

FIG. 3. Inhibition (% reduction from controls) of net phosphorus (Pi) uptake in 12-day-old cucumber seedlings as the net uptake of ferulic acid (FER) per g root dry weight increased. Seedlings were treated in nutrient solutions (pH 5.5; 0.5 mM Pi) containing 0.1–1.0 mM FER. Equation of regression model: % inhibition net Pi uptake = −40.95 + 4.82 (mg FER uptake/g root) − 0.05 (mg FER uptake/g root)^2; $P = 0.0127$; $R^2 = 0.39$.

TABLE 1. FERULIC ACID (FER) UPTAKE, PHOSPHORUS (Pi) UPTAKE, AND % INHIBITION Pi UPTAKE OF CUCUMBER SEEDLINGS AS MODIFIED BY Pi CONCENTRATION, TREATMENT SOLUTION pH, AND SEEDLING AGE (MEANS ± STANDARD ERROR)

	Treatment FER concentration (mM)	Net FER uptake (mg/g root)	Net Pi uptake (mg/g root)	Inhibition net Pi uptake (%)
Pi (mM)				
0.25	0.5	39.23 ± 1.80	1.17 ± 0.09	70.54 ± 2.37
0.5	0.5	39.58 ± 2.61	1.50 ± 0.12	52.43 ± 1.89
1.0	0.5	37.85 ± 3.39	2.18 ± 0.11	48.19 ± 2.70
Age (days)				
9	0.6	66.20 ± 5.51	2.09 ± .033	56.85 ± 6.75
12	0.6	46.78 ± 3.27	1.65 ± .004	58.77 ± 1.12
15	0.6	46.49 ± 6.02	2.08 ± 0.18	50.09 ± 4.42
pH				
4.5	0.5	35.31 ± 2.49	1.25 ± 0.23	69.10 ± 5.69
5.5	0.5	41.19 ± 1.97	2.38 ± 0.19	46.76 ± 4.25
6.5	0.5	28.24 ± 2.59	3.16 ± 0.23	28.61 ± 5.21

different from each other. Representative data for the effects of Pi concentration are shown for the 0.5 mM FER treatment group (Table 1). At each Pi concentration, the percent inhibition of net Pi uptake increased as the treatment FER concentration was increased. For Pi concentrations of 0.25, 0.5, and 1.0 mM, the regression models of percent inhibition of net Pi uptake by FER concentration had R^2 values of 0.82, 0.76, and 0.40, respectively (Figure 4). The regression model for percent inhibition of net Pi uptake by FER uptake per gram of root for the 0.25 mM Pi treatment had an R^2 value of 0.54 [% inhibition net Pi uptake = 24.88 + 1.11 (mg FER uptake/g root dry wt); $P = 0.0061$; $R^2 = 0.54$]; the regression models were not significant for the 0.5 and 1.0 mM Pi treatments.

Seedling Age. The relationship between net FER uptake and FER concentration was modified by seedling age (significant interaction; $P = 0.0045$). Net FER uptake per gram of root dry wt at a given FER concentration decreased as seedling age increased (Figure 5). The slopes were different between the 0.4 and 0.8 mM FER regressions, but not between the 0.4 and 0.6 or the 0.6 and 0.8 mM FER regressions. The percent inhibition of net Pi uptake was not affected by seedling age over the range of ages tested (9–15 days old). The regression model for percent inhibition of net Pi uptake by FER concentration had an R^2 of 0.47 compared to an R^2 value of 0.19 for the regression model for percent inhibition of Pi uptake by FER uptake per gram of root dry wt. Patterns were similar to previous experiments. The regression models for this experiment were as follows: % inhibition net Pi uptake = 36.75 + 25.91 (FER concentration); $P < 0.0001$, $R^2 = 0.47$; and % inhibition net Pi uptake = 33.75 + 0.47 (mg FER uptake/g root dry wt); $P = 0.002$, $R^2 = 0.19$.

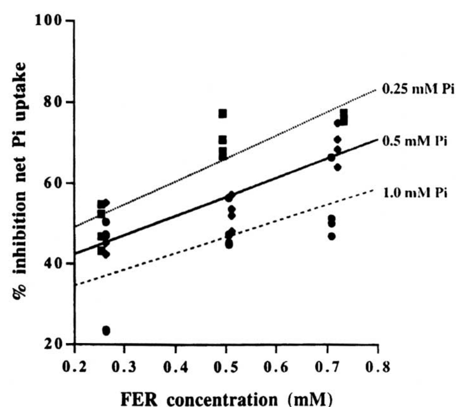

FIG. 4. Inhibition (% reduction from controls) of net phosphorus (Pi) uptake in 12-day-old cucumber seedlings treated in nutrient solutions (pH 5.5) containing 0.25–0.75 mM ferulic acid (FER) and 0.25–1.0 mM Pi. Data points: ■ = 0.25 mM Pi; ♦ = 0.5 mM Pi; ● = 1.0 mM Pi. Equations of regression models: 0.25 mM Pi – % inhibition net Pi uptake = 37.30 + 56.65 (FER concentration), $P < 0.0001$, $R^2 = 0.82$; 0.5 mM Pi – % inhibition net Pi uptake = 32.55 + 47.61 (FER concentration), $P = 0.0002$, $R^2 = 0.76$; 1.0 mM Pi – % inhibition net Pi uptake = 26.31 + 39.67 (FER concentration), $P = 0.0296$, $R^2 = 0.40$.

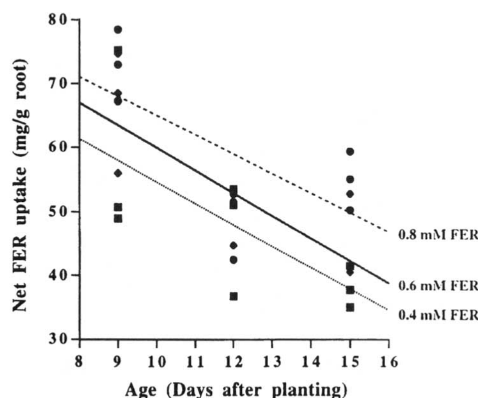

FIG. 5. Net uptake of ferulic acid (FER) by cucumber seedlings treated in nutrient solutions (pH 5.5) containing 0.4–0.8 mM FER as modified by seedling age. Data points: ■ = 0.4 mM FER; ♦ = 0.6 mM FER; ● = 0.8 mM FER. Equations of regression models: 0.4 mM FER – mg FER uptake/g root dry wt = 88.00 – 3.36(age), $P = 0.0347$, $R^2 = 0.49$; 0.6 mM FER – mg FER uptake/g root dry wt = 95.03 – 3.53 (age), $P = 0.0410$, $R^2 = 0.53$; 0.8 mM FER – mg FER uptake/g root dry wt = 94.97 – 3.02(age), $P = 0.0496$, $R^2 = 0.45$.

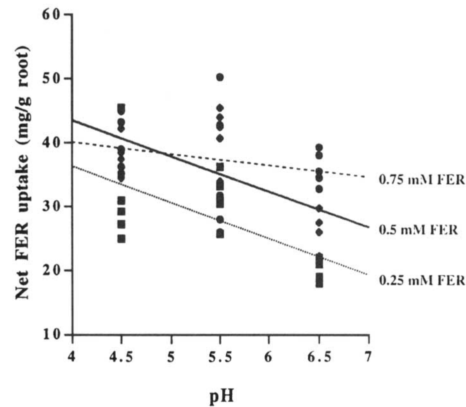

FIG. 6. Net uptake of ferulic acid (FER) by 12-day-old cucumber seedlings treated in nutrient solutions containing 0.25–0.75 mM FER as modified by treatment solution pH. Data points: ■ = 0.25 mM FER; ♦ = 0.5 mM FER; ● = 0.75 mM FER. Equations of significant regression models: 0.25 mM FER – mg FER uptake/g root dry wt = 58.85 – 5.68(pH), $P = 0.0082$, $R^2 = 0.43$; 0.5 mM FER – mg FER uptake/g root dry wt = 65.55 – 5.57(pH), $P = 0.0098$, $R^2 = 0.41$. The regression model for 0.75 mM FER was not significant.

pH. The relationship between net FER uptake and FER concentration was modified by pH (significant interaction; $P = 0.0025$). The effects of pH on net FER uptake varied with the concentration of FER in solution (Figure 6). Net FER uptake declined with increased pH for the 0.25 and 0.5 mM FER treatments (Table 1). The pH did not modify FER uptake for the highest concentration tested (0.75 mM FER). The slopes of the 0.25 and 0.5 mM FER regressions were different. Net Pi uptake per gram of root dry wt was not affected by pH for the control group (0 mM FER) over the pH range tested (4.5 to 6.5). The mean net Pi uptake of controls was 4.29 ± 0.15 mg/g root dry wt. The effect of FER concentration on percent inhibition of net Pi uptake was modified by pH (significant interaction; $P = 0.0178$) such that the inhibitory effects of FER decreased as the pH was increased. Representative data for the effects of pH are shown for the 0.5 mM FER treatment group (Table 1). The regression model for percent inhibition of net Pi uptake by FER concentration was not significant at pH 4.5 (Figure 7). At pH 5.5 and 6.5, the percent inhibition of net Pi uptake increased as the FER concentration was increased (slopes of these two regressions were different); the R^2 values for the linear models were 0.71 and 0.45, respectively. The regression models of percent inhibition of net Pi uptake by FER uptake per gram of root dry wt was not significant at pH 4.5 and 5.5. At pH 6.5, the regression model for percent inhibition of net Pi uptake by FER

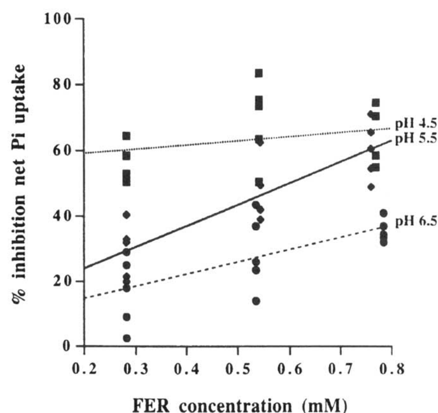

FIG. 7. Inhibition (% reduction from controls) of net phosphorus (Pi) uptake in 12-day-old cucumber seedlings treated in nutrient solutions (pH 4.5–6.5; 0.5 mM Pi) containing 0.25–0.75 mM ferulic acid (FER). Data points: ■ = pH 4.5; ♦ = pH 5.5; ● = pH 6.5. Equations of significant regression models: pH 5.5 – % inhibition net Pi uptake = 11.06 + 64.54 (FER concentration), $P = 0.0001$, $R^2 = 0.71$; pH 6.5 – % inhibition net Pi uptake = 6.83 + 37.40(FER concentration), $P = 0.0065$, $R^2 = 0.45$. The regression model for pH 4.5 was not significant.

uptake per gram root dry wt had an R^2 value of 0.34 [% inhibition net Pi uptake = −0.35 + 0.98 (mg FER uptake/g root dry wt); $P = 0.0226$; $R^2 = 0.34$].

Container Size. Container size did not affect net FER or Pi uptake. Uptake and inhibition patterns were similar to those seen in previous experiments. The regression model for net FER uptake by FER concentration was as follows: net FER uptake/g root dry wt = 0.21 + 147.75 (FER conc) – 113.58 (FER conc)^2; $P < 0.0001$; $R^2 = 0.85$.

The regression model for percent inhibition of net Pi uptake by FER concentration had an R^2 of 0.42: [% inhibition net Pi uptake = 5.90 + 62.55 (FER concentration); $P < 0.0001$; $R^2 = 0.42$]. The regression model for percent inhibition of net Pi uptake by FER uptake per gram of root dry wt was not significant.

Split-Root Experiment. The proportion of roots treated in FER did not affect the net Pi uptake of roots in the nutrient solution container, and the proportion of roots in nutrient solution did not affect the impact of FER on percent inhibition net Pi uptake for roots in the FER treatment (i.e., effects of FER were localized). For roots treated with FER (treatment container only), net FER uptake and percent inhibition net Pi uptake increased as the FER concentration was increased [net FER uptake/g root dry wt = 16.21 + 52.99 (FER concentration); $P = 0.0001$; $R^2 = 0.30$; percent inhibition net Pi uptake of treated roots = 7.13 + 79.79 (FER concentration); $P = 0.0001$; $R^2 = 0.46$)]. the model for per-

cent inhibition net Pi uptake of treated roots by FER uptake per gram of root was not significant.

When data were analyzed on a whole-plant basis, it was observed that, at any given FER concentration, the total net FER uptake per seedling increased as the proportion of roots exposed to FER increased. The percent inhibition of net Pi uptake per seedling increased as the product of proportion of root treated × FER concentration increased and as proportion of root treated × FER uptake per gram of treated root dry wt increased. The model for percent inhibition of total net Pi uptake per seedling by proportion of root treated × FER concentration had an R^2 of 0.56 compared to an R^2 of 0.28 for the model for percent inhibition of total net Pi uptake per seedling by FER uptake per gram of root. The linear equations for these models were as follows:

$$\% \text{ inhibition total net Pi uptake} = -1.60 - 85.59 \text{ (PFER} \times \text{CONCF)}; \quad P = 0.0000; R^2 = 0.56$$

$$\% \text{ inhibition total net Pi uptake} = -6.52 + 1.32 \text{ (PFER} \times \text{FER uptake/g root)}; \quad P = 0.0292; R^2 = 0.28$$

where PFER is the proportion of roots treated with FER and CONCF is the FER concentration.

DISCUSSION

The hypothesis that root uptake of allelopathic agents such as phenolic acids (e.g., ferulic acid) is a good estimator of dose was rejected. In nutrient culture, the inhibition of net Pi uptake was related more frequently and more consistently to the initial ferulic acid solution concentrations surrounding cucumber seedling roots than to ferulic acid uptake by these roots (i.e., models using FER uptake per gram of root as the dependent variable were not significant or had lower R^2 values and higher P values than models using FER concentration as the dependent variable). This was true for the range of Pi concentrations (0.25, 0.5, and 1.0 mM), seedling ages (9, 12, and 15 days old), proportion of root systems exposed to FER (7–100%), and volume of treatment solutions (20, 40, 60, and 80 ml) tested. It was also true for treatment solutions with pH values of 5.5 and 6.5. At pH 4.5, the third pH tested, inhibition of net Pi uptake was not related to either the initial ferulic acid concentration or to ferulic acid uptake because maximum inhibition was already observed for the lowest ferulic acid concentration tested (0.25 mM). These consistent findings provide strong circumstantial evidence that FER uptake is not a reliable indicator of dose. Particularly convincing evidence comes from the experiments where FER uptake at a given concentration was

modified by pH and seedling age; the observed Pi uptake inhibition did not follow the change in FER uptake, but rather continued to coincide more closely with the initial FER concentration in the solutions.

In designing these experiments we attempted to modify the nature and the relationships between cucumber roots (i.e., seedling age, root proportion treated) and ferulic acid (i.e., solution pH, container size, concentration). We hoped that such modifications would break the collinearity between root contact and/or root uptake of ferulic acid and ferulic acid inhibition of net Pi uptake by roots. To make the test more rigorous, we chose to use only ferulic acid concentrations that were linearly related to ferulic acid uptake. In four instances we succeeded in breaking this collinearity (i.e., regression models for percent inhibition net Pi uptake by FER concentration were significant but regression models for percent inhibition net Pi uptake by FER uptake were not significant). In five other instances we succeeded in modifying this collinearity (i.e., models for percent inhibition net Pi uptake by FER uptake had lower R^2 values and higher P values than models for percent inhibition net Pi uptake by FER concentration).

These data led us to suggest that root membrane contact, not uptake, is essential for the inhibitory activity of ferulic acid (i.e., simple phenolic acids). This suggestion is supported by the localized inhibitory effects of phenolic acids on growth, water utilization, and net Pi uptake observed in this study and in previous split-root experiments (Lyu and Blum, 1990; Lehman et al., 1994). Additional support is provided by the reversible nature of inhibitory activities of phenolic acids once they are removed from the root environment (Glass, 1973, 1974; Glass and Dunlop, 1974; Blum and Dalton, 1985; Baziramakenga et al., 1995), and in the rapid recovery of membrane potentials when phenolic acids are washed from membranes (Glass and Dunlop, 1974; Balke, 1985). In summary, the primary effect of phenolic acids on net Pi uptake appears to be a result of root membrane perturbations.

The pK_a of FER is 4.5; thus, at pH 4.5 approximately 50% of the FER molecules in solution would be protonated. At pH 5.5 and 6.5, only 10% and 1%, respectively, of FER molecules would be protonated. At a given FER concentration, the level of Pi uptake inhibition increased as pH was decreased, suggesting that it is the protonated form of FER molecules that interacts with the root surface. When the total number of molecules in solution was modified by container size, there were no significant effects on Pi uptake inhibition. Thus, it is likely that the concentration of protonated molecules, not the total number of protonated molecules surrounding roots, determines the level of root–FER interactions. This suggests that FER concentration in the treatment solution is a reliable predictor of allelochemical dose as long as the pH remains stable. It is likely that FER uptake was a less reliable indicator of dose in these experiments because uptake values represented the cumulative number of molecules that entered the plant roots over 5 hr. Depending on the mechanism of FER uptake (passive or

active) and the concentration gradient of FER inside the root versus outside in the solution, protonated FER molecules may be rapidly and continuously entering plant roots or may be in contact with the surface of root membranes for various time intervals before entering the root. Thus, the measured cumulative uptake values were not necessarily good indicators of the frequency and amount of membrane–FER contact that occurred during an experiment.

Caution must be used, however, in applying the results of this study to nature. In buffered nutrient culture systems, the concentration of FER is highly related to root contact and thus is a reliable indicator of dose. This is not necessarily true in soil systems due to other soil sinks (e.g., microbes, clays, organic matter), the reduced mobility of FER molecules, and the constant changes in FER concentrations resulting from activities of various sources and sinks in the soil. Thus, unless a soil system is at steady state, an unlikely occurrence in nature, estimates of soil solution concentrations of phenolic acids by soil extractions may not be closely related to the doses experienced by roots.

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CHEMICAL PROTEIN ANALYSIS: A COMPARISON OF KJELDAHL CRUDE PROTEIN AND TOTAL NINHYDRIN PROTEIN FROM WILD, TROPICAL VEGETATION

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Abstract—Various methods are available for measuring the protein content of vegetation. This paper reviews and tests some common methods in order to provide recommendations to wildlife ecologists and primatologists. The traditional Kjeldahl crude protein method, which requires the conversion factor of 6.25 (CP6.25), was compared to values obtained through total ninhydrin protein (NP) analysis and by calculating crude protein from a newly developed conversion factor of 4.3 (CP4.3). The NP analysis gave values that were not significantly different from the CP4.3 values, and both were significantly lower than the CP6.25 values. An additional method was compared (available CP), which is CP6.25 values corrected by subtracting the acid-detergent lignin-bound crude protein. Comparisons were also made between CP4.3 and the available CP. These two methods correct the protein values differently, and theoretically CP4.3 corrects more severely, as explained in the text, but in some situations they may be correcting for the same chemical category of unavailable nitrogen. For fruits and flowers these values were not significantly different. For leaves the results were not so clear-cut. The Ugandan leaves ($N = 42$), Indonesian mature leaves ($N = 40$), Zimbabwe leaves ($N = 24$), and northern USA leaves ($N = 11$) were significantly different, and the CP4.3 always corrected more severely. The rest of the leaf sets (Belize, $N = 68$; Zaire, $N = 36$; Sumatra, $N = 10$; St. Catherine's Island, Georgia,

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USA, $N = 37$; and southern USA, $N = 18$) did not give a significant difference between CP4.3 and available CP. The choice of which analysis method to use (NP or CP4.3 versus available CP) depends on whether it seems reasonable to severely penalize all nonprotein nitrogen, considering the animal species being studied, or whether removing only the lignin-bound nitrogenous compounds is sufficient. Overall, the traditional 6.25 conversion factor is too large a conversion value for most wild vegetation; crude protein corrected only for lignin-bound protein is probably more accurate.

Key Words—Plant protein content, total nitrogen, amino acid analysis, nitrogen-protein conversion.

INTRODUCTION

Quantifying the protein content of diets consumed by free-ranging, wild animals is extremely important for describing the nutritional ecology of a given species or to compare the diets of sympatric species. There is a need for an accurate assay of protein, particularly in the analysis of plants, which is also quick and economical. Various methods are available: (1) Individual amino acid analysis (AOAC, 1984; Aurand et al., 1987) is very accurate but costly and time consuming. (2) Total ninhydrin protein (Marks et al., 1985; Makkar et al., 1988; Barbehenn, 1995) borrows from the procedure for individual amino acid analysis, is quicker, and reasonably accurate, but does not give individual amino acid content. (3) The Lowry method (Lowry et al., 1951; Amory and Schubert, 1987) is accurate on animal products but susceptible to many interfering compounds found in plants. (4) The Bradford method (Jones et al., 1989) measures only intact proteins, missing free amino acids and small polypeptides. (5) The biuret method (Aurand et al., 1987) measures only peptides/polypeptides greater than three units. (6) The Kjeldahl nitrogen method (Pierce and Haenisch, 1947; AOAC, 1984) directly and accurately measures the total nitrogen content of a sample. A conversion factor is then used to estimate crude protein.

The Kjeldahl nitrogen method frequently is used because it is an acceptable compromise between simplicity, economics, and accuracy. Nevertheless, several authors have disputed the conversion factor of 6.25 commonly used to convert Kjeldahl N into crude protein value (Jones, 1931; Milton and Dintzis, 1981; Herbst, 1986; Dintzis et al., 1988; Handley et al., 1989; Izhaki, 1992, 1993; Yeoh and Wee, 1994). The new conversion factors determined by these authors, who used amino acid analysis to calculate the percentage of N in wild plant proteins, are invariably lower than 6.25. Based on combining values for wild temperate and/or tropical plants, mostly ripe fruit, and mature leaves (122 samples in total), the mean conversion factor is calculated to be 4.3 ± 0.6 (Table 1). Leaves, fruit, and fleshy vegetables are mostly lacking from previous evalu-

TABLE 1. CONVERSION FACTORS FOR DIFFERENT PLANT PARTS

Plant part	Conversion factor	*N*	Reference
Flowers	3.9 ± 0.7	3	Milton and Dintzis (1981)
Ripe fruit pulp	4.0 ± 0.8	31	Milton and Dintzis (1981), Herbst (1986), Izhaki (1993)
Dicots			
Young leaf	4.2 ± 0.5	3	Milton and Dintzis (1981)
Mature leaf	4.4 ± 0.4	63	Yeoh and Wee (1994), Milton and Dintzis (1981)
Monocots, mature leaf	4.4 ± 0.7	22	Yeoh and Wee (1994), Handley et al. (1989)
Weighted mean	4.3 ± 0.6	122	

ations of conversion factors (Jones, 1931; Watt and Merrill, 1963; Dintzis et al., 1988). Fruit tended to have a slightly lower value compared to leaves (Table 1). This new conversion factor of 4.3 is what will be used (CP4.3) in this paper to compare to total ninhydrin protein (NP).

This report gives the results of the first comparison made between (NP) (Marks et al., 1985) and Kjeldahl crude protein (CP) calculated with the 6.25 conversion (CP6.25), and with a conversion factor of 4.3, averaged from selected literature. In addition, acid-detergent insoluble crude protein (ADCP) is used to correct the CP6.25 values and to compare those values to ninhydrin protein. The Marks et al. (1985) ninhydrin method was chosen because it is a quicker, simpler procedure for quantifying total amino acids, without actually counting or identifying individual amino acids.

Comparisons are made between ADCP, which is primarily lignin-bound protein (Pichard and Van Soest, 1977; Krishnamoorthy et al., 1982), although it may include tannin-bound protein and the tannin content of the samples. Tannins have been suggested as one reason why the 6.25 correction factor is too high (Milton and Dintzis, 1981).

A review of these methods is important at this time because there is increased interest in quantifying the nutrient contents of wild diets. Various authors have offered their own conversion factors as being more appropriate, or their own analysis methods as being more precise. This paper explains how some of the different methods or conversion factors are related and will help researchers interpret and compare their results with those already published from different methods. An important application of this work is when one is attempting to support captive animals on wild diets or commercial diets meant to mimic wild diets. It is important to accurately estimate available nutrients in order to maintain the health of the animals.

METHODS AND MATERIALS

The primary samples were collected in Africa at Kibale Forest National Park, Uganda, as part of projects for studying the nutritional ecology of chimpanzees, red-tail monkeys, blue monkeys, and mangabeys. A second set of samples was collected during projects on the ecology of *Pithecia* spp. and *Chiropotes* spp. in Venezuela. All samples were air-dried in the field (20–30°C, 24–48 hr, in a room kept dry enough to prevent molding; see Conklin-Brittain et al., 1998). The Ugandan samples included 63 ripe pulps, 13 ripe seeds, 31 unripe pulps, 11 unripe seeds, seven mature leaves, and 46 young leaves. The Venezuelan samples included 27 ripe seeds, two unripe seeds, six ripe fruit, four young leaves, and three mature leaves. The samples were shipped to Harvard and dried in a forced-air oven at 40°C in the Harvard Anthropology Department Nutritional Ecology Laboratory. They were then ground to pass through a 20-mesh screen in a Wiley mill and the following analyses were performed. A third set of data comparing CP values versus available crude protein (CP minus ADCP) values came from samples analyzed at the laboratory in the Wildlife Conservation Society (WCS) in New York. The staff at WCS perform the procedures in a manner essentially identical to those performed by the Harvard personnel.

Kjeldahl Crude Protein

The method used here is traditionally used to analyze domestic livestock feed (AOAC, 1984). Tecator Kjeltec digestion and distillation units were used to perform the analysis. The digestion mix contained Na_2SO_4 and $CuSO_4$. The distillate was collected in 4% boric acid containing methyl red and methylene blue indicators. It was then titrated with 0.1 N HCl. The determined percentage of nitrogen on a dry matter basis is then multiplied by 6.25 or by 4.3.

Acid-Detergent Insoluble Crude Protein

The acid-detergent fiber procedure was performed on 1-g samples in duplicate (Goering and van Soest, 1970, as modified by Robertson and Van Soest, 1980). However, after refluxing, the fiber and solution were filtered through Whatman No. 54 instead of Gooch crucibles. The resulting fiber residue and filter paper were folded to prevent loss of fiber, dried at 100°C, and then placed in the Kjeldahl digestion tubes. The Kjeldahl procedure described above was performed. The filter paper does not contribute any contaminating nitrogen. Available crude protein (available CP) was calculated by subtracting ADCP (also calculated as $\times 6.25$) from CP6.25.

Modified Ninhydrin Method for Total Proteins

The procedure of Marks et al. (1985), as modified by Barbehenn (1995), was used. This procedure measures all amino acids at once and is subject to the same constraints facing individual amino acid analysis (Aurand et al., 1987). It excludes nitrogen not found in amino acid form, which is referred to as nonprotein nitrogen.

In duplicate, 20 mg of dried, ground plant sample were boiled in 10 ml 6 N HCl at 110°C for 22 hr. The test tubes were cooled and 8.4 ml 28% NaOH were added. The solution was filtered through Whatman No. 1. An aliquot of 0.1 ml sample was mixed with 1 ml ninhydrin reagent. The ninhydrin reagent was prepared as follows: For reagent A, sodium citrate (2.941 g) and 0.08 g stannous chloride were mixed in 50 ml water, and the pH adjusted to 5.0. For reagent B, ninhydrin (triketohydridene hydrate) (2 g) and 50 ml ethylene glycol monomethyl ether (amino acid analysis grade) were mixed, and reagent A was mixed with reagent B.

The tubes containing samples were capped and the contents boiled for 20 min. Five milliliters of 50% isopropanol was added, and the tubes were votexed and cooled for 15 min. The absorbance was read at 570 nm. The standard was bovine serum albumin (Sigma No. A-7030), treated the same way as the samples but diluted with distilled water 1 : 1 after filtering, before aliquots were taken.

Tannins

Condensed tannin or proanthocyanidin (CT) content was measured by the proanthocyanidin test of Bate-Smith (1975) as modified by Porter et al. (1986). The total tannin or protein binding ability was determined from the radial diffusion (RD) method of Hagerman (1987). Quebracho was the standard used for both assays. Results were given as percentage of quebracho units (QU), rather than as percentage of dry matter or organic matter (OM). This means that if all the tannins had been quebracho, the values would have been on an organic matter basis. However, we assume none of the tannins were, in fact, identical to quebracho, and since no universal standard exists for tannins as a group, results were expressed as percentage of quebracho units.

All Ugandan results are presented on an organic matter basis to eliminate potentially varying amounts of ash (minerals) in the different samples. Organic matter is calculated as (100 − % ash) × % dry matter (at 100°C). The Venezuelan samples are on a room temperature dry matter basis because sample sizes were generally too small to perform the 100°C dry matter and ash analysis. The third data set (from the Wildlife Conservation Society) for available CP (availCP), CP calculated with 6.25, and CP calculated with 4.3 are included to further explore the relationship between these two methods, which potentially may give similar values. These results are on a 100°C dry matter basis.

Unpaired, two-tailed t tests were used to compare the different chemical methods. Simple fit regressions also were performed.

RESULTS

Appendices 1 and 2 contain the data used for the following comparisons. Appendix 3 contains some of the data from the Wildlife Conservation Society, and the rest of that data set has been published, as indicated in Table 4 below. Regarding the Ugandan data set, CP6.25 gave significantly higher values than ninhydrin protein (NP) (Table 2). No other comparisons, however, were statistically different. There were no differences in NP compared to availCP, or to

TABLE 2. COMPARISONS AMONG CRUDE PROTEIN CALCULATED WITH 6.25 (CP6.25), NINHYDRIN PROTEIN (NP), AVAILABLE CRUDE PROTEIN (CP6.25 MINUS ACID-DETERGENT INSOLUBLE CP6.25) (AVAILCP), AND CRUDE PROTEIN CALCULATED WITH 4.3 (CP4.3)

% OM[a]						
CP6.25	NP	AvailCP	CP4.3	r^2	P t test	N
Ugandan samples						
18.1 ± 10.8	13.1 ± 7.2			0.76	0.001	141
20.0 ± 11.2	14.2 ± 7.8			0.79	0.001	79
	14.2 ± 7.8	16.5 ± 11.8		0.79	0.15	79
	13.1 ± 7.2		12.5 ± 7.4	0.76	0.45	141
	14.2 ± 7.8		13.8 ± 7.7	0.79	0.72	79
		14.6 ± 11.1	12.3 ± 7.4	0.97	0.06	125
		16.5 ± 11.8	13.8 ± 7.7	0.97	0.08	79
Plant part comparisons						
leaves		24.1 ± 10.9	19.1 ± 6.6		0.01	42
fruit		9.8 ± 7.7	9.0 ± 5.0		0.39	81
leaves	19.4 ± 7.2	25.0 ± 10.7			0.01	38
fruit	9.7 ± 5.3	9.3 ± 7.7			0.79	43
% DM[a]						
CP6.25	NP	AvailCP	CP4.3			
Venezuelan samples						
7.9 ± 4.7	5.9 ± 3.6			0.68	0.02	43
	5.9 ± 3.6		5.5 ± 3.2	0.67	0.59	43

[a]% OM = percentage of organic matter; % DM = percentage of dry matter.

TABLE 3. COMPARISONS AMONG ACID-DETERGENT INSOLUBLE CRUDE PROTEIN (ADCP), CONDENSED TANNINS (CT), AND TOTAL TANNINS (RD), WITH SAMPLES FROM UGANDA[a]

ADCP (% OM)	CT (% QU)	RD (% QU)	N	r^2
3.3 ± 2.1	5.7 ± 9.8		125	0.009
3.3 ± 2.1		6.5 ± 9.0	125	0.001

[a]% OM = percentage of organic matter; % QU = percentage of quebracho tannin organic matter.

CP4.3, and availCP compared to CP4.3. The results listing an N of 79 represent a subsample of 79 samples for which all chemical analyses were done and include data for all four variables. The conclusions were the same for the unequal sample sizes and the equal samples sizes. All comparisons were highly correlated ($P <$ 0.01 for all of the r^2 values in Table 2), especially the CP4.3 versus availCP comparisons, with an r^2 value of 0.97.

Values for CP4.3 and availCP were not significant for the whole data set. However, when leaves (both young and mature) were considered separately from the other plant parts (pulp, seed, husks), the values were different for leaves but not for fruit (Table 2: leaves: $t = -2.559$, $P = 0.01$; fruit: $t = -0.864$, $P = 0.39$). Separating the comparisons between NP and availCP according to leaf or fruit parts also reveals that for leaves this comparison was significantly different ($t = -2.67$, $P = 0.01$) but not for fruit ($t = 0.27$, $P = 0.79$).

The Venezuelan samples also followed the same pattern. However, availCP could not be determined because the sample sizes were too small, and there was not enough sample material for the ADCP analysis.

There was no significant correlation between ADCP and CT, nor between ADCP and RD (Table 3). Tannin measures could not predict the ADCP value or vice versa. A t test could not be performed because the ADCP was on a percentage organic matter basis and the tannins on a percentage Quebracho units basis.

The results from the Wildlife Conservation Society laboratory (Table 4) show similar trends to the Ugandan and Venezuelan samples. The fruit and flower CP4.3 versus availCP comparisons were not significantly different. The leaves sometimes were not different, but three sets were significantly different (Indonesian mature leaves, Zimbabwe leaves, and northern USA leaves).

DISCUSSION

There are many different methods available to quantify nitrogen or protein, especially for analyzing foods of animal origin. However, most of them have shortcomings for the nutritional analysis of plant samples. For example, the

TABLE 4. VALUES FROM WILDLIFE CONSERVATION SOCIETY NUTRITION LABORATORY FOR CP6.25, AVAILCP, AND CP4.3[a]

Plant part	N	CP6.25	availCP	CP4.3	AvailCP/CP4.3 t-test P value	Reference
Belize samples						Silver (1997)
Young leaves	45	24.0 ± 7.2	18.5 ± 7.8	16.5 ± 5.0	0.15	
Mature leaves	23	17.9 ± 4.2	13.8 ± 4.2	12.3 ± 2.9	0.15	
Fruits	18	8.0 ± 3.4	5.0 ± 3.4	5.5 ± 2.4	0.61	
Flowers	20	16.6 ± 5.4	11.2 ± 4.7	11.4 ± 3.7	0.89	
Zaire samples						Hart (unpubl. data), Molloy (unpubl. data)
Fruits, buds	10	8.8 ± 2.8	4.9 ± 2.2	6.1 ± 2.0	0.23	
Leaves	36	15.3 ± 4.2	10.0 ± 4.7	10.6 ± 2.9	0.56	
Indonesian samples						Yeager et al. (1997)
Fruit and flowers	8	7.8 ± 3.5	5.0 ± 2.5	5.3 ± 2.4	0.77	
Mature leaves	40	8.4 ± 1.7	4.6 ± 1.6	5.8 ± 1.2	0.001	
Sumatra samples						Appendix 3
Leaves	10	12.0 ± 3.0	8.8 ± 3.6	8.3 ± 2.0	0.69	
Zimbabwe samples						Dierenfeld et al. (1995)
Leaves	24	16.3 ± 2.8	12.9 ± 4.3	10.2 ± 3.0	0.015	
USA samples						
Leaves						
St. Catherine's Island, GA samples	37	12.1 ± 5.1	8.9 ± 5.7	8.4 ± 3.5	0.59	Appendix 3
Southern USA samples	18	15.2 ± 8.9	12.2 ± 9.6	10.4 ± 6.2	0.50	Dierenfeld et al. (submitted) Graffam et al. (1997)
Northern USA samples	11	16.3 ± 2.8	13.4 ± 3.7	11.2 ± 1.9	0.02	Nijboer and Dierenfeld (1996)

[a]See Table 2 for abbreviations. All values are as a percentage of dry matter.

Lowry method is sensitive but nonspecific (Bensadoun and Weinstein, 1976), reacting with interfering compounds such as phenolics found in crude plant extracts (Marks et al., 1985; Jones et al., 1989). The Bradford method stains and measures only whole, intact proteins (Compton and Jones, 1985; Jones et al., 1989), thus ignoring the nutritional value of free amino acids, small peptides, and acid amides (Milton and Dintzis, 1981). The biuret method also measures only peptides larger than dipeptides, thus missing free amino acids, dipeptides, and acid amides (Aurand et al., 1987). These three compound categories are particularly common in young plant material where growth is rapid (Milton and Dintzis, 1981), and they should not be discriminated against when discussing the nutritional value of the nitrogenous compounds in plant material.

While there are additional chemical shortcomings to the above procedures, amino acid analysis after standard acid hydrolysis and treatment with ninhydrin also has its procedural problems, in particular the total loss of tryptophan due to acid hydrolysis (Williams, 1982). In addition, proline and hydroxyproline give a yellow reaction instead of a blue/purple reaction and are not well detected at the wavelength used by the spectrophotometric method used (Aurand et al., 1987). Nevertheless, most automatic amino acid analyzers use ninhydrin to measure the individual amino acids, including those in the reports used here to calculate the 4.3 conversion factor, so a ninhydrin total protein method is the most logical method for our comparisons here.

Traditionally the percentage of nitrogen determined by the Kjeldahl method is multiplied by 6.25, a conversion factor resulting primarily from animal protein studies (Milton and Dintzis, 1981; Jones, 1931), in which it was found that 16% was the mean percentage of N in animal protein (100/16 = 6.25). Coincidentally, the grains fed livestock generally also contain 16% N (Jones, 1931). The dispute regarding the use of 6.25 for plant foods has two parts, one of which is that not all plant proteins contain 16% N (Jones, 1931; Watt and Merrill, 1963; Dintzis et al., 1988), and thus these should be calculated with a different correction factor. Yet, despite the length of this dispute, even in domestic foods (Jones, 1931), 6.25 has remained the standard. In meat most of the nitrogen present is in amino acids incorporated into the meat proteins, and there is much less non-amino acid nitrogen compared to plants. The non-amino acid nitrogen content of plants can increase the percentage of nitrogen contained in plant "protein" from the 16% typical in meat up to 23% in plants. The second problem is that plants contain varying amounts of nonproteinaceous N from secondary plant compounds or unavailable protein N due to variable binding with lignin or tannins (Pichard and Van Soest, 1977; Swain, 1979). Most animal protein is available and easily digested.

The results presented here clearly indicate that CP6.25 overestimates the protein value of wild plant samples. The fact that NP is not significantly different from the recalculated CP4.3 is supporting evidence that the ninhydrin method does duplicate the much more complicated method of calculating total protein content

by individual amino acid analysis. This factor of 4.3 was averaged from a data set heavily dominated by mature leaves. The Venezuelan collection is dominated by seeds (Appendix 2). It has been suggested that 5.3 would be a more appropriate conversion factor for temperate climate, domestic seeds (Dintzis et al., 1988) although, based on our results, not for tropical seeds.

As previously mentioned, plants contain protein that has been bound up by lignin in the cell wall. The determination of acid-detergent insoluble crude protein (ADCP) was developed by ruminant nutritionists interested in a quick, easy method to correct for lignin-bound and therefore completely unavailable plant protein. This fraction would be equally unavailable to nonruminant animals, since lignin is refractive to digestion by virtually all animals (Van Soest, 1994). This is not, however, the only nitrogen-containing fraction that is not part of the available/digestible protein fraction. Besides protein and amino acids, nitrogen is also present in ammonia molecules, in all of the intermediate compounds potentially resulting from amino acid breakdown or synthesis (general N metabolism), in nucleic acids, in alkaloids (in plants), in chitin (in insects), etc., and is generally referred to as nonprotein nitrogen (NPN).

The efficiency of use of NPN varies among animal species. It is generally considered available to pregastric fermenters because the microbial populations in the foregut convert NPN into microbial protein. The host animal then digests the microbes, and the protein contained in the microbial bodies, as they are swept into the acid stomach and small intestine (Van Soest, 1994). Simple-stomached mammals have not been studied in great detail, but since they have no foregut and therefore do not host microbes in any organ preceding the acid stomach, they do not have the advantage that pregastric fermenters have. The same amount of NPN that a ruminant can utilize, for example, as urea or ammonia, can be toxic to a simple-stomached animal. Nevertheless, during the synthesis of nonessential amino acids in the liver, free ammonia is required by all animals. In addition, some animals use NPN in a detoxification capacity, such as the desert tortoise eating high potassium plants (Oftedal, 1998). The degree to which NPN should be eliminated from a protein calculation has not been definitively determined for simple-stomached species.

The NP and CP4.3 values are theoretically correcting for the same chemical fraction, which is all NPN, since both values result from measuring only amino acid nitrogen. Acid-detergent bound protein (ADCP) is probably a subfraction of NPN, although this cannot be said definitively because it is unknown whether any lignin-bound protein is measured by NP or CP4.3. It probably is not measured because the acid extraction step is probably not strong enough to separate bound protein (Dintzis et al., 1988). Consequently it is not surprising when, as for the Ugandan leaves, the availCP gives significantly higher values compared to CP4.3. One could call availCP the optimist's protein value, and NP or CP4.3 the pessimist's protein value. AvailCP has only removed the lignin-bound pro-

tein, leaving the rest of the NPN, which has a more ambiguous nutritional fate. Some NPN consists of potentially useful compounds (Milton and Dintzis, 1981; Martinez del Rio, 1994), but the nutritional fate of all the different components of the NPN has not been determined. The NP or CP4.3 values assume that all of the NPN is of no nutritional value.

The lack of correlation between ADCP and the two tannin fractions is somewhat surprising. Chemically, tannins are in the same family as lignins. Many tannins stay in the acid-detergent residue with the lignin (Van Soest, 1994). One would expect them to be there with their proteins still bound to them. However, only about 25% of the Ugandan samples had condensed tannin values above 5% QU (Appendix 1), and 60% of the samples were 2% QU or less. It is possible that with this relatively low tannin collection of plants, the tannins were not contributing consistently to the ADCP value. It is possible that preextracting the ADCP with neutral detergent would wash out the more soluble tannins, leaving a more predictive fraction. However, tannins are also extremely diverse in their reactions to pH and to different protein types (Martin et al., 1985; Hagerman and Butler, 1980, 1981). They are probably less universally predictive of protein quality than is ADCP.

In conclusion, the 6.25 conversion factor is too high for determining the protein content of wild vegetation. Nevertheless, there are many types of studies where the original CP6.25 is adequate. Since it is simply a mathematical calculation, values reported in the literature can easily be converted into CP4.3, assuming one is interested only in amino acids and protein. It is important that authors specify which conversion factor they have used if their results are from Kjeldahl crude protein, so that the reader can recalculate if necessary. The availCP procedure is probably the more realistic determination, given that most animals probably use some of the NPN present. However, that method is more labor intensive and expensive, requiring more steps and more plant material.

These results also confirm that papers publishing values of either NP or CP (6.25 or 4.3) can be compared with each other; the NP values can be assumed to be CP4.3 values. The lack of consensus regarding leaves and the comparability of availCP versus NP or CP4.3 may stem from the fact that leaves are the most metabolically active part of a plant and probably contain more NPN, some of which the leaves are actively using to synthesize amino acids. The choice of methods depends on how much one wants to penalize this nonprotein nitrogen fraction. Feeding trials and labeled-nitrogen studies would help resolve this question.

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APPENDIX 1[a]

Species	CP6.25 (% OM)	CP4.3 (% OM)	NP (% OM)	ADCP (% OM)	AvailCP (% OM)	CT (% QU)	RD (% QU)
Cambium							
Celtis africana	14.3	9.8		1.1	13.2	0.3	0.0
Fruit husk/skin							
Casearea engleri	15.6	10.8		9.7	5.9	0.1	1.4
Monodora myristica	9.4	6.4	7.0	5.1	4.3	20.9	13.6
Mimusopa bagshawei	8.9	6.2	6.8			0.7	3.2
M. bagshawei	8.0	5.5		4.8	3.2	2.3	0.3
M. bagshawei	11.9	8.2		3.2	8.7	3.1	0.2
Pterygota mildbraedii	15.0	10.3	7.7			62.5	13.2
Mean	11.5	7.9	7.2	5.7	5.5	14.9	5.3
SD	3.2	2.2	0.5	2.8	2.4	24.6	6.4
Ripe Pulp							
Casearea engleri	15.9	11.0		2.9	13.0	0.2	0.0
Celtis africana	22.6	15.5		1.8	20.8	0.0	0.0
C. africana	17.7	12.2	14.6			0.0	0.0
C. durandii	18.3	12.6	14.4	1.9	16.4	0.1	0.0
C. durandii	18.7	12.8		2.0	16.6	0.1	0.0
C. durandii	19.8	13.6		3.1	16.7	0.0	0.0
Chaetacme aristata	17.6	12.1	10.4	3.8	13.9	22.8	27.8
C. aristata	28.7	19.8	17.9	2.7	26.0		
C. aristata	28.8	19.8	14.1	2.7	26.0	56.0	9.0
Diospyros abyssinica	7.6	5.3	5.7	1.2	6.4	1.5	19.8
D. abyssinica	19.0	13.1	6.2				
Fagaropsis angolensis	16.4	11.3		1.3	15.1	0.3	0.4
F. angolensis	30.3	20.8	21.7			0.1	1.3
Ficus asperifolia	13.9	9.6		1.5	12.4	0.1	0.0
F. brachylepis	5.2	3.6	4.0	4.2	1.0	1.2	0.0
F. brachylepis	8.7	6.0	9.5			1.4	
F. brachylepis	7.4	5.1	7.0			0.8	
F. brachylepis	8.2	5.7	8.2	1.8	6.5	0.4	2.9
F. conraui	6.4	4.4	25.2			8.3	3.4
F. conraui	7.8	5.3		3.4	4.4	0.8	
F. cyathistipula	4.4	3.0		2.8	1.5	0.4	0.0
F. exasperata	23.4	16.1	11.0			0.0	
F. exasperata	25.4	17.4	18.4	2.0	23.4	0.0	0.0
F. exasperata	10.2	7.0	6.9			1.9	9.7
F. exasperata	26.2	18.0		4.1	22.1	0.0	1.4
F. mucuso	4.4	3.0		2.0	2.4	0.4	0.0
F. natalensis	6.1	4.2		1.9	4.2	2.0	2.1
F. natalensis	6.7	4.6	6.3	4.4	2.3	0.1	1.2
F. ovata	5.4	3.7		2.4	3.0	8.0	16.1
F. sansibarica	9.7	6.7		2.0	7.7	2.1	3.3
F. sansibarica	9.6	6.6	8.4			4.8	

APPENDIX 1[a] CONTINUED

Species	CP6.25 (% OM)	CP4.3 (% OM)	NP (% OM)	ADCP (% OM)	AvailCP (% OM)	CT (% QU)	RD (% QU)
F. sansibarica	12.1	8.3	9.5			2.6	18.9
F. saussureana	7.6	5.2	8.4				6.1
F. saussureana	7.7	5.3	7.1			1.5	6.0
F. saussureana	9.5	6.6	8.8	4.0	5.5	1.2	2.1
F. saussureana	8.1	5.5		5:8	2.3	1.1	1.7
Linociera johnsonii	10.2	7.0	9.0	6.7	3.5	0.7	4.1
Macaranga sp.	7.4	5.1	7.3	2.0	5.4	9.6	48.2
Mimusops bagshawei	7.2	5.0	5.3			0.5	3.2
M. bagshawei	6.0	4.1	3.9			3.2	0.2
M. bagshawei	7.6	5.2	4.3	2.7	4.9	0.9	0.0
M. bagshawei	8.3	5.7	4.5	3.3	5.0	1.7	0.2
M. bagshawei	9.1	6.3		4.2	4.9	2.0	0.2
M. bagshawei	6.5	4.5		7.2	−0.7	3.0	0.3
M. bagshawei	6.8	4.6	3.7	3.5	3.3	2.0	0.2
M. bagshawei	7.7	5.3	3.9	4.4	3.3	1.0	0.2
M. bagshawei	9.6	6.6	5.4			1.4	0.2
Monodora myristica	6.8	4.7	5.3	2.8	4.1	10.6	10.4
Myrianthus arboreus	12.8	8.8	12.1	2.9	9.8	14.7	15.3
Pancovia turbinata	10.8	7.5	9.7	3.1	7.7	2.4	18.2
Parinari excelsa	7.1	4.9	6.6	3.6	3.5	9.4	7.0
Pseudospondias microcarpa	10.8	7.5	7.1	5.9	5.0	1.7	8.4
Psycotria capensis	10.1	6.9	9.3			3.3	10.3
Strychnos mitis	9.4	6.5	7.1	3.8	5.6	0.0	0.0
Symphonia globulifera	6.9	4.7	5.5	5.0	1.9	13.7	16.2
Teclea nobilis	17.8	12.3		1.9	15.9	0.1	0.0
Uvariopsis congensis	21.0	14.5	18.2	2.7	18.3	0.0	10.8
Mean	12.2	8.4	9.3	3.2	9.0	3.7	5.7
SD	6.9	4.8	5.1	1.4	7.5	8.5	9.1
Ripe seed							
Celtis durandii	21.6	14.8		2.7	18.9	0.0	0.0
Diospyros abyssinica	8.5	5.8		1.1	7.3	2.2	6.3
Ficus exasperata	15.5	10.7	9.3			0.1	
F. exasperata	15.6	10.7	11.2			0.0	
F. exasperata	6.4	4.4	4.7			6.3	
F. natalensis	6.1	4.2	7.0			1.5	
F. sansibarica	9.7	6.7		1.4	8.3	1.3	9.1
F. sansibarica	7.2	4.9	6.9			8.6	
Mimusops bagshawei	4.8	3.3		1.5	3.3	0.2	0.0
M. bagshawei	4.5	3.1		2.8	1.6	3.2	3.8
Pterygota mildbraedii	17.3	11.9	14.1			43.9	19.9
P. mildbraedii	23.6	16.2	9.1	8.8	14.7	31.7	15.9
Symphonia globulifera	4.9	3.4	6.7			37.8	28.2

APPENDIX 1[a] CONTINUED

Species	CP6.25 (% OM)	CP4.3 (% OM)	NP (% OM)	ADCP (% OM)	AvailCP (% OM)	CT (% QU)	RD (% QU)
Mean	11.2	7.7	8.6	3.1	9.0	10.5	10.4
SD	6.7	4.6	3.0	2.9	6.6	16.0	10.1
Whole Figs (seed + pulp)							
Ficus exasperata	19.5	13.4	13.9				
F. exasperata	19.8	13.6	12.7				
F. exasperata	20.3	14.0	16.1				
F. exasperata	19.2	13.2	16.0				
F. exasperata	18.9	13.0	15.8			0.1	0.4
F. exasperata	14.3	9.8	16.0			0.1	0.4
F. exasperata	18.4	12.7	16.1			0.1	0.4
F. natalensis	6.5	4.4	6.6				
F. natalensis	6.7	4.6	9.4				
F. natalensis	6.2	4.3	6.2			1.0	3.3
F. natalensis	7.4	5.1		4.8	2.6	0.8	1.7
F. natalensis	7.6	5.3	7.4			0.4	
F. sansibarica	10.0	6.9	9.2			6.9	
F. sansibarica	26.3	18.1	17.6				
Mean	14.4	9.9	12.5	4.8	2.6	1.3	1.2
SD	6.8	4.7	4.2			2.5	1.3
Unripe pulp							
Celtis africana	29.3	20.1	27.2	2.4	26.9	0.0	0.0
C. durandii	26.8	18.5		1.8	25.0	0.4	2.7
C. durandii	27.3	18.8	18.7	1.8	25.5	0.2	1.8
Chaetacme aristata	17.9	12.3	15.9	3.8	14.1	29.3	36.4
Dasylepis eggelingi	19.8	13.6	14.7				
Diospyros abyssinica	10.0	6.9	8.2	1.9	8.1	2.0	6.2
Fagaropsis angolensis	27.3	18.8	21.2	2.4	24.0	0.1	0.4
Ficus exasperata	22.0	15.1		2.9	19.1	0.2	0.3
F. exasperata	25.4	17.5	14.2			0.1	0.4
F. natalensis	7.1	4.9	6.8			0.6	4.5
F. natalensis	5.6	3.9	6.3			0.3	0.8
F. ovata	5.8	4.0	4.8	2.3	3.6	21.6	18.3
F. sansibarica	8.7	6.0		2.0	6.7	1.7	21.6
F. sansibarica	10.5	7.3	7.5			2.9	7.9
F. sansibarica	10.4	7.2	7.5	3.1	7.4	5.1	12.2
F. saussureana	9.5	6.5	9.4	3.5	6.0	3.1	8.2
Linociera johnsonii	8.6	5.9	7.8	5.3	3.3	0.0	2.7
Macaranga sp.	8.8	6.0	8.0	2.6	6.2	1.9	34.0
Mimusopa bagshawei	7.5	5.1	5.4			9.8	0.0
M. bagshawei		0.0	6.7			0.6	
M. bagshawei	8.1	5.6	3.3	2.2	5.9	7.3	0.3
M. bagshawei	9.7	6.7		4.1	5.7	2.4	0.2

APPENDIX 1[a] CONTINUED

Species	CP6.25 (% OM)	CP4.3 (% OM)	NP (% OM)	ADCP (% OM)	AvailCP (% OM)	CT (% QU)	RD (% QU)
Myrianthus arboreus	12.6	8.7	11.4	4.0	8.6	39.9	36.1
Pancovia turbinata	16.0	11.0	13.9	3.0	13.0	11.5	17.1
Parinari excelsa	8.5	5.9	7.5	4.7	3.8	8.5	6.5
Pseudospondias microcarpa	7.6	5.2	6.7	5.2	2.4	2.1	14.8
Psycotria capensis	12.6	8.7	12.6			6.6	19.6
Strychnos mitis	6.8	4.7		2.0	4.8	0.0	0.0
Symphonia globulifera	6.6	4.6	6.3			15.3	18.6
Teclea nobilis	17.3	11.9		1.4	15.9	0.0	0.0
Uvariopsis congensis	19.0	13.1		2.5	16.6	0.2	0.0
Mean	13.8	9.2	10.5	2.9	11.5	5.8	9.4
SD	7.5	5.4	5.8	1.1	8.2	9.5	11.5
Unripe seed							
Celtis durandii	13.2	9.1	8.7	4.2	9.1	0.0	0.0
Diospyros abyssinica	9.0	6.2		1.1	7.9	16.1	11.8
Ficus exasperata	15.0	10.3	9.1			0.0	0.0
F. natalensis	5.7	3.9	6.0			0.5	5.0
F. natalensis	9.0	6.2	9.9			0.3	0.0
Linociera johnsonii	9.5	6.5	7.8	6.1	3.4	0.0	3.8
vine #126	23.0	15.8		1.4	21.6	4.3	6.4
Milletia dura	32.5	22.4		1.4	31.1	12.8	12.9
Mimusopa bagshawei	7.5	5.1		2.7	4.8	4.6	7.7
Strychnos mitis	5.4	3.7		0.5	5.0	0.0	0.0
William's fruit #164	16.8	11.5		3.1	13.7	1.6	0.0
Mean	13.3	9.2	8.3	2.6	12.1	3.6	4.3
SD	8.3	5.7	1.5	1.9	9.7	5.6	4.9
Mature leaf							
Cassipourea ruwenzoriensis	26.5	18.2	17.4	2.5	23.9	26.5	16.0
Celtis africana	36.8	25.3	16.8	2.9	33.9	0.0	0.0
C. durandii	36.2	24.9	18.7	2.1	34.1	0.4	0.0
Chaetacme aristata	28.8	19.8	16.9			5.4	14.9
Ficus asperifolia	23.5	16.2	19.0	2.5	21.0	0.4	0.4
F. exasperata	25.2	17.3	6.5	2.1	23.1	0.4	0.0
Neoboutonia macrocalyx	22.2	15.3	17.7	2.3	19.9	19.6	13.4
Mean	28.4	19.6	16.1	2.4	26.0	7.5	6.4
SD	5.9	4.1	4.3	0.3	6.4	11.0	7.9
Young leaf							
Celtis africana	39.1	26.9	30.3			4.5	3.5
Diospyros abyssinica	26.7	18.4	19.4			3.6	5.1
Funtumia africana	29.5	20.3	22.0			17.9	10.6
F. africana	31.3	21.6	21.5			15.8	9.7

APPENDIX 1[a] CONTINUED

Species	CP6.25 (% OM)	CP4.3 (% OM)	NP (% OM)	ADCP (% OM)	AvailCP (% OM)	CT (% QU)	RD (% QU)
Monodora myristica	45.1	31.1	28.7	1.2	43.9	0.0	0.0
M. myristica	45.6	31.4	39.9	2.0	43.6	0.1	0.0
Markhamia platycalyx	11.7	8.1	9.7	4.8	6.9	0.0	0.9
Aningeria altissima	17.6	12.1	15.4	3.4	14.1	41.9	19.9
Bosqueia phoberos	19.5	13.4	13.6	3.4	16.1	23.2	15.2
B. phoberos	18.9	13.0	12.9	3.4	15.5	15.5	9.2
Cassipourea ruwenzoriensis	21.5	14.8	13.1	4.9	16.5	9.8	2.6
Celtis africana	41.5	28.5	34.0	2.8	38.7	0.0	0.0
C. africana	38.5	26.5	28.7	2.0	36.5	0.2	0.3
C. africana	39.7	27.3	19.5	2.1	37.6	0.1	0.0
C. durandii	31.7	21.8	24.0	2.3	29.4	0.1	1.7
C. durandii	35.3	24.3	11.8	1.9	33.4	4.3	0.0
Chaetacme aristata	30.9	21.3		4.2	26.8	24.5	15.3
C. aristata	31.6	21.7	18.5			3.5	8.0
Diospyros abyssinica	24.9	17.1	18.7	1.7	23.2	2.0	25.1
Dombeya mukole	27.6	19.0	19.2	2.1	25.5	6.2	7.0
D. mukole	35.4	24.3	27.3	2.5	32.8	15.8	10.9
Ficus asperifolia	21.2	14.6	17.2	1.6	19.5	0.4	0.4
F. exasperata	31.1	21.4	21.6			0.1	0.9
F. exasperata	31.5	21.7	29.8	1.4	30.1	0.3	0.9
F. exasperata	30.8	21.2	22.5	1.7	29.1	0.5	0.4
F. exasperata	36.3	25.0	13.1			3.2	0.0
F. sansibarica	11.9	8.2	11.3	1.3	10.6	2.4	12.3
Funtumia africana	19.3	13.3		8.4	10.9	8.8	5.0
F. africana	22.2	15.3		3.2	19.1	10.2	16.3
Markhamia platycalyx	18.9	13.0	13.0	7.2	11.7	0.0	3.2
M. platycalyx	29.8	20.5	20.0	7.0	22.8	0.0	1.2
Milletia dura	29.6	20.3	19.9	5.0	24.5	2.3	6.3
M. dura	41.6	28.6	26.1	1.6	40.0	14.3	12.7
Mimusops bagshawei	10.4	7.2	8.1	4.2	6.2	19.6	17.2
Monodora myristica	31.6	21.8	17.8			0.8	0.0
M. myristica	26.8	18.5	18.2	2.3	24.5	0.1	0.0
Olea welwitschii	14.1	9.7	11.0	9.3	4.8	0.2	0.8
O. welwitschii	23.5	16.2	16.8	14.4	9.1	0.4	7.9
Parinari excelsa	13.5	9.3		8.4	5.1	22.3	19.2
Premna angolensis	45.2	31.1	24.3	6.2	39.0	0.2	1.7
Strombosia scheffleri	29.7	20.4	19.2	2.9	26.8	0.3	0.0
Trichillia splendida	23.7	16.3	17.6	2.8	20.9	16.4	10.2
Vanqueria apiculata	29.6	20.4	26.0	2.0	27.6	0.2	1.7
V. apiculata	37.0	25.5	25.3	1.9	35.1	0.8	7.6
Markhamia platycalyx	18.3	12.6	13.4			0.1	0.8

APPENDIX 1[a] CONTINUED

Species	CP6.25 (% OM)	CP4.3 (% OM)	NP (% OM)	ADCP (% OM)	AvailCP (% OM)	CT (% QU)	RD (% QU)
M. platycalyx	11.0	7.6	8.0			0.0	0.9
Mean	27.9	19.2	19.7	3.8	23.8	6.4	5.9
SD	9.7	6.7	7.2	2.9	11.5	9.3	6.7
Total mean	17.1	11.7	13.1	3.3	14.6	5.7	6.4
Total SD	10.3	7.1	7.2	2.1	11.1	10.4	8.6

[a]CP6.25 = crude protein calculated with the 6.25 conversion factor; CP4.3 = crude protein calculated with the 4.3 conversion factor; NP = total ninhydrin protein; ADCP = acid-detergent bound crude protein; availCP = available protein = CP6.25 minus ADCP; CT = condensed tannins; RD = radial diffusion total tannins; % OM = percentage of organic matter; % QU = percentage of quebracho units. Samples of the same species from different areas of the forest are reported separately.

APPENDIX 2[a]

Species	NP (% DM)	CP6.25 (% DM)	CP4.3 (% DM)	CT (% QU)	RD (% QU)
Seeds, no coats					
Chrysophyllum lucentifolium	6.9	8.0	5.5	0.6	2.4
C. lucentifolium	9.9	14.3	9.9	0.0	0.0
Lepidocordia punctata	8.0	10.8	7.5	0.3	1.6
Peltogyne floribunda	7.2	6.6	4.6	6.8	11.8
Mean	8.0	10.0	6.9	1.9	4.0
SD	1.4	3.4	2.3	3.3	5.3
Seed coats					
Chrysophyllum lucentifolium	4.0	2.8	1.9	0.7	0.0
Pradosia caracasana	0.4	2.4	1.7	8.4	8.0
P. caracasana	0.5	2.4	1.7	6.1	7.4
P. caracasana	0.4	2.4	1.7	10.5	8.7
Peltogyne floribunda	6.4	7.9	5.4	8.0	29.6
Mean	1.3	2.5	1.7	6.4	6.0
SD	1.8	0.2	0.1	4.2	4.1
Whole ripe seeds					
Connarus venezuelanus	2.5	5.3	3.7	4.2	
Melicoccus bijugatus	9.5	9.8	6.7	0.9	0.4
M. bijugatus	8.9	8.6	5.9	1.0	0.0
Pradosia caracasana	0.7	3.1	2.2	7.4	10.1
P. caracasana	0.7	3.0	2.0	9.9	10.1
P. caracasana	0.8	3.7	2.5	10.0	10.1

APPENDIX 2[a] CONTINUED

Species	NP (% DM)	CP6.25 (% DM)	CP4.3 (% DM)	CT (% QU)	RD (% QU)
Maprounea guianensis	11.9	16.4	11.3	1.1	0.0
Lepidocordia punctata	9.1	11.0	7.6	0.0	1.2
Oryctanthus alveolatus	8.4	9.6	6.6	2.4	4.9
Brosimum alicastrum	7.7	8.4	5.8	0.1	1.6
Strychnos mitscherlichii	7.3	7.0	4.8	0.0	0.0
S. mitscherlichii	5.7	5.6	3.9	0.0	1.6
Copaifera sp.	8.4	9.8	6.7	1.5	6.5
Pradosia caracasana	1.8	0.5	0.4	8.0	5.0
P. caracasana	1.6	1.4	1.0	7.7	7.5
P. caracasana	1.6	1.4	1.0	7.6	7.1
P. caracasana	1.5	1.7	1.2	8.4	7.1
Actinostemon schomburgkii	8.8	11.4	7.8	6.9	12.5
Mean	5.4	6.5	4.5	4.3	5.0
SD	3.9	4.4	3.0	3.8	4.2
Unripe whole fruit					
Coccoloba striata	5.3	7.5	5.2	2.9	19.1
Unripe seeds					
Maprounea guianensis	12.1	13.6	9.4	1.0	2.8
Pradosia caracasana	0.4	3.1	2.2	6.8	12.7
Mean	6.3	8.4	5.8	3.9	7.8
SD	8.3	7.4	5.1	4.1	7.0
Mesocarp					
Chrysophyllum lucentifolium	6.3	8.2	5.7	5.9	6.5
C. lucentifolium	6.0	7.9	5.4	1.3	0.8
Melicoccus bijugatus	8.8	11.4	7.8	0.1	0.0
Mean	7.0	9.1	6.3	2.4	2.4
SD	1.6	1.9	1.3	3.1	3.5
Whole, ripe fruit/berries					
Coccoloba striata	6.0	6.1	4.2	2.2	16.2
Morinda tenuiflora	10.0	11.7	8.0	0.2	2.0
Amaioua corymbosa	8.2	9.1	6.2	2.7	4.5
Mean	8.1	9.0	6.2	1.7	7.6
SD	2.0	2.8	1.9	1.3	7.6
Young leaf					
Maytenus sp.	6.4	10.0	6.9	12.8	
Maytenus sp.	4.5	11.9	8.2	14.2	
Maytenus sp.	4.8	12.8	8.8	15.9	
Coccoloba fallax	13.9	15.4	10.6	10.7	30.8
Mean	7.4	12.5	8.6	13.4	
SD	4.4	2.2	1.5	2.2	
Mature leaf					
Maytenus sp.	3.5	8.3	5.7	8.6	
Coccoloba fallax	8.3	9.1	5.3	6.2	32.5

APPENDIX 2[a] CONTINUED

Species	NP (% DM)	CP6.25 (% DM)	CP4.3 (% DM)	CT (% QU)	RD (% QU)
C. fallax	6.7	7.9	5.4	4.1	14.6
Mean	7.5	8.5	5.8	5.2	23.6
SD	1.1	0.9	0.6	1.5	12.7
Total mean	5.9	7.7	5.3	5.0	7.8
SD	3.6	4.1	2.8	4.4	8.5

[a]NP = total ninhydrin protein; CP6.25 = crude protein calculated with the 6.25 conversion factor; CP4.3 = crude protein calculated with the 4.3 conversion factor; CT = condensed tannins; RD = radial diffusion total tannins; % DM = percentage dry matter; % QU = percentage of quebracho units. Samples of the same species from different areas of the forest are reported separately.

APPENDIX 3[a]

Species	Plant part (% DM)	CP6.25 (% DM)	AvailCP (% DM)	CP4.3 (% DM)
St. Catherine's Island, Georgia, USA				
Arundinaria sp.	L	11.0	9.7	7.6
Arundinaria gigantea	L	10.8	9.3	7.4
A. gigantea	L	9.4	8.1	6.5
Bumelia tenax	L	10.7	9.0	7.3
Carya sp.	L	13.0	5.0	9.0
Celtis laevigata	L	12.7	11.7	8.8
C. laevigata	L	15.9	14.2	10.9
C. laevigata	L	11.9	10.2	8.2
C. occidentalis georgiana	L	13.6	12.0	9.3
Ilex opaca	L	8.1	5.4	5.6
I. vomitoria	L	3.4	1.8	2.3
Magnolia grandiflora	L	7.1	4.6	4.9
Melia azedarach	L	21.8	20.1	15.0
Myrica cerifera	L	14.6	6.7	10.1
M. cerifera	L	12.9	5.3	8.9
M. cerifera	L	12.1	6.5	8.4
M. cerifera	L	12.1	7.5	8.3
Parthencissus quinquefolia	L	14.0	12.3	9.7
Parthenocissus quinquefolia	L	13.2	11.3	9.1
Persea borbonia	YL	13.3	11.7	9.1

APPENDIX 3[a] CONTINUED

Species	Plant part (% DM)	CP6.25 (% DM)	AvailCP (% DM)	CP4.3 (% DM)
P. borbonia	ML	7.7	3.8	5.3
P. borbonia	L	9.2	5.7	6.3
P. borbonia	L	11.4	6.6	7.8
Quercus virginiana	L	10.6	7.3	7.3
Q. virginiana	L	10.8	5.2	7.4
Q. virginiana	L	9.7	6.6	6.7
Q. virginiana	L	9.7	6.9	6.7
Q. virginiana	YL	30.3	29.9	20.8
Q. virginiana	L	10.0	5.8	6.9
Smilax sp.	L	9.7	6.6	6.7
Tillandsia usneoides	L	4.2	2.6	2.9
Unknown sp.	L	7.6	6.7	5.3
Vitis rotundifolia	L	16.2	12.8	11.1
V. rotundifolia	L	14.3	10.2	9.8
V. rotundifolia	ML	9.8	3.1	6.8
V. rotundifolia	L	10.2	4.9	7.0
Zanthoxylum clava-herculis	L	25.9	23.6	17.8
Mean		12.1	8.9	8.3
SD		5.1	5.7	3.5
Leaves eaten by Sumatran rhino in Sumatra				
Species 1	leaf	6.9	2.1	4.7
Species 2	leaf	13.0	11.1	8.9
Species 3	leaf	12.1	9.6	8.3
Species 4	leaf	9.4	5.2	6.5
Species 5	leaf	13.3	7.7	9.2
Species 6	leaf	18.1	15.5	12.5
Species 7	leaf	10.7	8.9	7.4
Species 8	leaf	13.6	9.2	9.4
Species 9	leaf	10.4	7.3	7.2
Species 10	leaf	12.7	11.4	8.7
Mean		12.0	8.8	8.3
SD		3.0	3.6	2.1

[a]L = leaf; YL = young leaf; ML = mature leaf; % DM = percentage of dry matter; CP6.25 = crude protein calculated with the 6.25 conversion factor; CP4.3 = crude protein calculated with the 4.3 conversion factor; availCP = available crude protein = CP6.25 minus acid-detergent bound crude protein. Samples of the same species from different areas of the forest are reported separately.

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THE RESPONSE OF *Phytoseiulus persimilis* TO SPIDER MITE-INDUCED VOLATILES FROM GERBERA: INFLUENCE OF STARVATION AND EXPERIENCE

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Abstract—When leaves of the ornamental crop *Gerbera jamesonii* are damaged by the spider mite *Tetranychus urticae*, they produce many volatile compounds in large quantities. Undamaged gerbera leaves produce only a few volatiles in very small quantities. In the headspace of spider mite-damaged gerbera leaves many terpenoids are present, comprising 65% of the volatile blend. In addition, a number of nitrogen containing compounds, such as oximes and nitriles, are produced.

We studied the attraction of *P. persimilis* to the volatiles from spider mite-damaged gerbera leaves and how attraction is affected by starvation and previous experience. *Phytoseiulus persimilis* that were reared on spider mites (*T. urticae*) on Lima bean were not attracted to spider mite-induced volatiles from gerbera. Starvation did not influence the predator's response to these volatiles. In contrast, predators that were reared on spider mites on gerbera leaves were strongly attracted to volatiles from spider mite-infested gerbera. This was found also for predators that originated from a culture on spider mite-infested bean and were offered six days of experience with spider mites on gerbera leaves.

Key Words—Herbivore-induced synomones, infochemicals, semiochemicals, learning, biological control, prey location, behavior, Acarina, *Phytoseiulus persimilis, Tetranychus urticae, Gerbera jamesonii.*

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INTRODUCTION

Phytoseiulus persimilis Athias-Henriot is a specialist predator of the herbivorous spider mite *Tetranychus urticae* Koch. Once it is present in an environment with an abundant presence of prey it gives rise to an exponentially growing population that can increase up to 57% per day (Krips et al., 1999). This capacity for population increase of the predator is much higher than that of its prey, which leads to rapid extermination of the herbivore population (Sabelis, 1985a).

Unlike many other species of predatory mites that can survive on alternative food sources, *P. persimilis* feeds almost exclusively on spider mites from the genus *Tetranychus* (Sabelis, 1985a). Hence, the ability to find patches where these spider mites are present is a matter of life or death for this predator. Any behavioral characteristic that would enable this predator to locate such patches from a distance would clearly be adaptive.

Several species of host plants respond to damage by spider mites with the production of volatiles that attract *P. persimilis* (Sabelis and van de Baan, 1983; Sabelis et al., 1984; Dicke, 1988a; Dicke and Sabelis, 1988; Dicke et al., 1990a, 1998; Sabelis and van der Weel, 1994; Sabelis and Afman, 1994). *Phytoseiulus persimilis* uses those odors to find leaves with colonies of its prey. Once *P. persimilis* is present on such leaves, the speed at which they find prey colonies is enhanced by odors from the damaged spot (Garms et al., 1998). Predators do not leave a prey patch until all prey is exterminated locally. This is most likely the result of arrestment in response to volatiles from damaged plants (Sabelis and van der Meer, 1986; Sabelis and Afman, 1994). The arrestment of the predators in a prey patch has a major influence on the population growth of the predators as well as their prey (van Baalen and Sabelis, 1995), which indicates the importance of the behavioral response to the volatiles.

Volatiles that are produced by plants in response to herbivore damage vary between plant species, between plant cultivars and also between leaves of different age (Dicke et al., 1990b; Takabayashi et al., 1990; 1991, 1994a,b; Turlings et al., 1993; Loughrin et al., 1995; see Takabayashi and Dicke, 1996 and Dicke et al., 1998, for reviews on variability of spider mite-induced plant volatiles). Since *T. urticae* is a herbivore with a very wide range of host-plant species (Sabelis, 1985b), a predator like *P. persimilis* is faced with a large variation in volatile information that indicates the presence of prey. It is very unlikely that *P. persimilis* has a fixed response to all these volatile blends.

The response of the predators varies largely with host-plant species (Dicke and Sabelis, 1988; Takabayashi and Dicke, 1992; Takabayashi et al., 1994b) and an initially low response can be enhanced by starvation, experience, or selection. For example, the predators are only attracted towards spider mite-damaged pear leaves after starvation (Dicke and Sabelis, 1988). The response to spider mite-damaged cucumber leaves increased significantly after the predators were

given several days of experience with spider mites on cucumber (Dicke et al., 1990c; Takabayashi and Dicke, 1992; Takabayashi et al., 1994b). Furthermore, with selection for several generations on high- or low-responding predators, it has been possible to establish lines that have a significantly different response from the original population. This indicates that, besides phenotypic plasticity, a genetic component determines the predators' response towards spider mite-induced volatiles (Margolies et al., 1997).

Based on our current knowledge, we formulate the following hypothesis regarding the foraging behavior of *P. persimilis*. Predators are arrested in a prey patch by spider mite-induced volatiles. Local extermination of the spider mites will decrease the production of volatiles by the plant and increase the starvation level of the predators. This will increase the response of predators to volatiles from a wider range of host plants. When a predator finds a prey patch on a plant of a new species, the initially low response to the spider mite-induced volatiles will increase due to experience. This will keep the predators in the new prey patch until all prey is exterminated.

Phytoseiulus persimilis is commonly and successfully used for biological control of *T. urticae* in vegetable crops in greenhouses. In ornamental crops, where aesthetic damage cannot be tolerated, biological control is usually more difficult. The ornamental gerbera (*Gerbera jamesonii*) is a positive exception to this rule, since gerbera cutflowers are sold without leaves, which allows some damage on leaves. Spider mites usually only damage leaves, which makes biological control of spider mites possible on gerbera (van de Vrie, 1985).

The present paper reports on the production of spider mite-induced volatiles by gerbera and the attraction of *P. persimilis* towards these volatiles. Furthermore, we investigated whether the attraction is affected by starvation or experience in a spider mite patch on gerbera. With the results of this study the hypothesis on the foraging behavior of *P. persimilis* will be evaluated.

MATERIALS AND METHODS

Plant Material. Gerbera (*Gerbera jamesonii*) plants (cv. Sirtaki) were obtained from a commercial gerbera grower, Terra Nigra bv, The Netherlands. They were subsequently grown in a greenhouse at the Laboratory of Entomology (Wageningen University, The Netherlands) at 20–30°C, 50–70% relative humidity, and a photoperiod of at least 16 hr of light. High-pressure mercury lamps switched on when the light intensity outside dropped below 150 watt/m^2 and switched off when it increased above 250 watt/m^2. Plants used for the experiments were 6–12 months old. Only the youngest, fully unfolded leaves were used in experiments.

Spider Mites. Spider mites (*Tetranychus urticae*) were collected from a commercial gerbera greenhouse at Mijdrecht, The Netherlands, in the spring of

1994 and were subsequently reared in our laboratory on gerbera cultivar Sirtaki. The spider mite culture was kept under the same conditions as the gerbera plants.

Predatory Mites. Predatory mites (*Phytoseiulus persimilis*) were originally obtained from Entocare CV, The Netherlands, a commercial mass rearing company for biological control. In our laboratory they were reared on spider mites (*T. urticae*) on either Lima bean leaves or leaves of the gerbera cultivar Sirtaki, depending on the experiment. Predators that were reared on spider mites on Lima bean had been kept on this plant species for many generations. Predators that were reared on spider mites on gerbera were kept on this plant species for at least two generations. The predators were kept in closed Petri dishes of 9 cm diameter and were offered pieces of leaves with spider mites three times a week. Once a week, a new colony was started by transferring five gravid female *P. persimilis* from each Petri dish to new dishes. The Petri dishes were kept in a climate room at 23 ± 1°C, 60 ± 5% relative humidity, and a photoperiod of 16L : 8D. For the predatory mite culture on bean leaves, regular Petri dishes were used. The predatory mite culture on gerbera leaves was kept in Petri dishes with an opening of 2.7 cm diameter that was covered with fine-mesh gauze.

Incubation of Leaves. The youngest, fully unfolded leaves were picked and placed with their petioles in glass vials containing tap water. To obtain spider mite damage, approximately 75 adult female *T. urticae* were placed on each leaf. Leaves were kept for 7 days at 23 ± 1°C, over 65% relative humidity, and a photoperiod of 16L : 8D. Control leaves without spider mites were kept for seven days under the same conditions as the treated leaves.

Experiment 1: Volatiles Produced by Undamaged Gerbera Leaves and Leaves with Spider Mite Damage—Collection and Analysis of Headspace Volatiles. Leaves were placed with their petioles in a 200-ml glass vial containing tap water. The vial with leaves was then placed in a 5-liter glass jar with a 10-cm-ID opening. The jar was closed with a lid that contained two openings. A glass tube was inserted through one of the openings to be used as air inlet. The glass tube nearly reached the bottom of the jar. An airstream was generated in the flask at 450 ml/min. Before reaching the inlet, the air was cleaned through silica gel, molecular sieves, and activated charcoal, following the procedure described by Takabayashi et al. (1991). Air was passed through the system for 1 hr prior to volatile collection, in order to remove all contaminants from the jar. Subsequently a Pyrex glass tube (160 × 6.0 mm OD, 3 mm ID) containing 90 mg Tenax-TA was connected to the outlet of the jar and air was purged for 15 min (6.75 liter) through this tube in order to collect the volatiles present in the jar.

The number of gerbera leaves used for volatile collection depended on the treatment. For collection of headspace volatiles from leaves that were damaged by spider mites, we used five to six leaves (= ±30 g) for each sample. For collection of headspace volatiles from undamaged gerbera leaves, we used 10 leaves for each sample. We collected five headspace samples from leaves with spi-

der mite damage and four samples from undamaged leaves. For each sample a different set of leaves was used. The volatiles from the Tenax tubes were analyzed by GC-MS, with the Thermdesorption Cold Trap Unit as inlet on the gas chromatograph as described in Mattiacci et al. (1994). The column used was a Supelcowax 10 fused silica capillary column, 60 m × 0.25 mm ID, 0.25-μm film thickness, with helium as carrier gas at an initial linear velocity of 22 cm/sec. The oven temperature was raised from 40°C to 270°C at 4°C/min.

Experiment 2: Response of P. persimilis *to Gerbera Volatiles and Influence of Starvation Level and Rearing History.* Ten undamaged control leaves and 10 leaves with spider mites were placed in separate 2-liter jars that contained a small amount of water to prevent the leaves from desiccating. The leaves were placed with their stems in the water. The jars were connected to a closed system Y-tube olfactometer, described in more detail by Takabayashi and Dicke (1992). Adult female *P. persimilis* were individually introduced into the olfactometer onto an iron wire running through the center of the olfactometer glass tube and running parallel to the tube walls. Predators were placed at the base of the Y-tube and had to choose between two olfactometer arms. Air with volatiles from spider mite-damaged leaves was led through one of the arms and air from clean leaves through the other arm. The observation of a predator ended when the animal reached the far end of one of the olfactometer arms. Observations lasted for no more than 5 min per individual predator. Predators that had not reached the end of one of the olfactometer arms within this time were excluded from the statistical procedure. This happened in only four cases out of a total number of 297 predators.

Two cultures of predatory mites that originated from the same source (see above) were used, one reared on Lima bean with spider mites and one on gerbera leaves with spider mites. Adult female predators from each culture were divided into two groups. One group was starved for 3 hr and the other group was starved for 24 hr prior to the start of the olfactometer test. Predators that were starved for 3 hr were placed individually in empty Eppendorf vials. Predators that were starved for 24 hr were placed individually in Eppendorf vials that contained a small piece of moist cotton wool to prevent the predators from desiccating. When predators of different treatments were tested on the same day (which can be read from Figure 2 below), we used the same set of gerbera leaves for all treatments. In these cases the predators from the different treatments were introduced alternatingly into the olfactometer. After five predators from each treatment were tested in the Y-tube olfactometer, the connections of the odor sources to the two arms of the olfactometer were interchanged. The numbers of replicates per treatment are indicated in Figure 2 below.

To test whether the predators were attracted to one of the volatile sources, for each replicate experiment a χ^2 test was performed on numbers from each predator culture and each starvation level separately. For each of the four treatments, the total numbers of predators that went to each volatile source were

added and a χ^2 test was performed on these numbers as well. To test whether the response was affected by rearing history, we added the numbers of both starvation levels and performed a 2×2 contingency test.

Experiment 3: Influence of Previous Experience on Response of P. persimilis *to Gerbera Volatiles.* We used the same Y-tube olfactometer set-up as described for experiment 2. From the predatory mite culture on spider mite-infested bean leaves, we separated two groups of adult females. One group was kept for six days on spider mite-infested bean leaves in regular Petri dishes of 9 cm diameter. The other group was kept for a total of six days on spider mite-infested gerbera leaves. They were kept in Petri dishes of 9 cm diameter with an opening of 2.7 cm diameter that was covered with fine-mesh gauze. Three days after these subcultures were started, all adult females were transferred to fresh leaves with spider mites. This was done to make sure that the experiment was performed with the individuals that originated from the stock culture on spider mite-infested bean leaves and not with their offspring.

For each replicate 20 predators from both subcultures were used and were introduced alternatingly into the Y-tube olfactometer. The connections of the odor sources to the two arms of the olfactometer were interchanged after five predators from each subculture were tested. The expriment was repeated three times.

To test whether the predators were attracted to one of the volatile sources, for each replicate experiment a χ^2 test was performed on numbers from each predator subculture separately. To test whether the response from the two predator subcultures differed, we added all numbers for each subculture separately and performed a 2×2 contingency test.

RESULTS

Experiment 1: Volatiles Produced by Undamaged Gerbera Leaves and Leaves with Spider Mite Damage. The gas chromatogram of headspace volatiles of undamaged gerbera leaves (Figure 1A) shows several peaks. However, the largest peaks are impurities from the collection system, such as siloxanes from silicone rubber seals. Although we used fewer leaves per sample for leaves with spider mite damage, the number of volatiles present in the sample is much larger and the volatiles are produced in higher quantities (Figure 1B).

We found considerable variation in total peak area in our gas chromatograms, which is partly due to the fact that not all samples were taken at the same time. Therefore, we calculated for each compound the relative peak area as a percentage of the total volatile production per sample rather than the average absolute peak area. Samples of control leaves and treated leaves were taken over the same time-span.

The compounds that are most prevalent in the headspace of undamaged gerbera leaves are 1-butanol, 4,8-dimethyl-1,3(*E*), 7-nonatriene and (Z)-3-hexen-1-

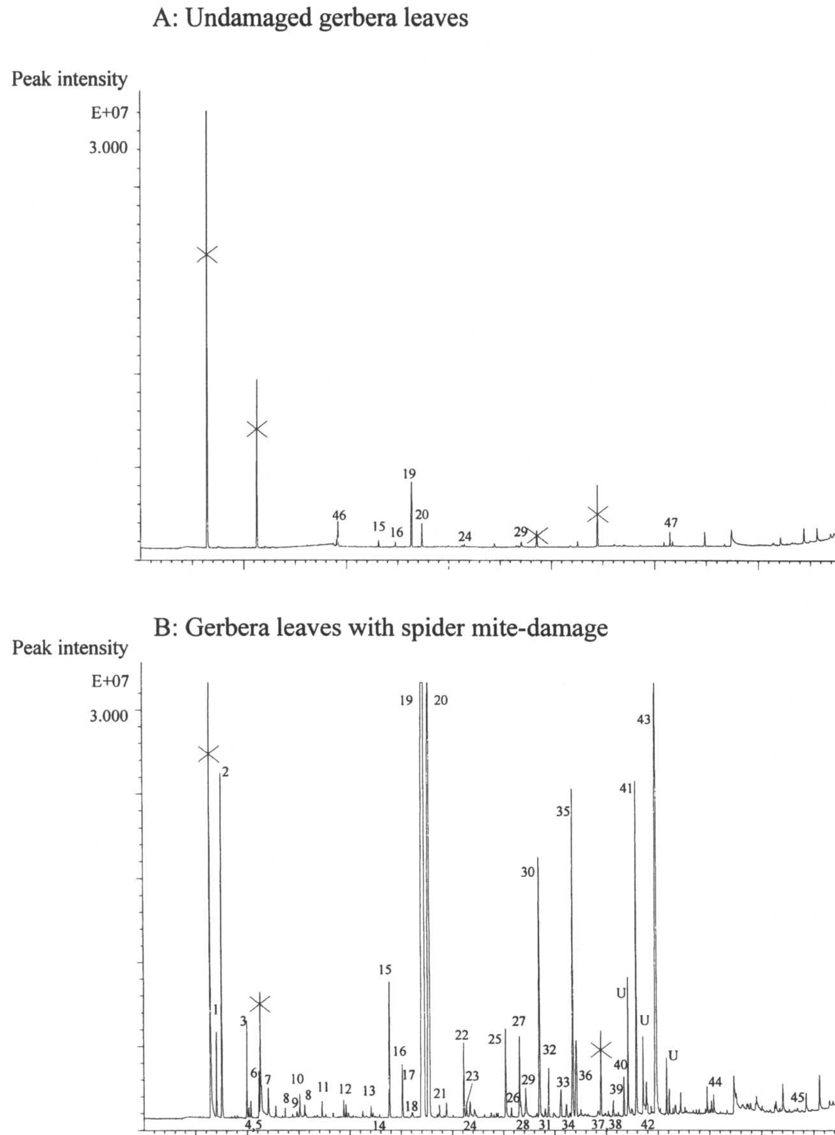


FIG. 1. Representative examples of gas chromatograms of headspace samples taken from undamaged gerbera leaves (A) and gerbera leaves with spider mite damage (B). Peaks with a cross are impurities from the collection system. Both chromatograms are plotted on the same scale. Numbers correspond with numbers from Table 1; only peaks that represent >0.05% of the total volatile blend are numbered. U = unidentified compound.

yl acetate (Table 1). The headspace of spider mite-damaged gerbera leaves is dominated by the compounds (*Z*)-3-hexen-1-yl acetate, 2-butanone, and a large number of terpenoids, among which 4,8-dimethyl-1,3(*E*),7-nonatriene, 4,8,12-trimethyl-1,3(*E*),7(*E*),11-tridecatetraene, (*E*)-β-ocimene, and (*E*,*E*)-α-farnesene are produced in the largest quantities (Table 1). Furthermore, several nitrogen-containing compounds, such as nitriles and oximes are produced by spider mite-damaged gerbera, but are absent in the headspace of undamaged gerbera leaves.

Experiment 2: Response of P. persimilis *to Gerbera Volatiles and Influence of Starvation Level and Rearing History.* The predatory mites that were reared on spider mite-infested bean leaves and were starved for 3 hr prior to the experiment had only a weak response to spider mite-induced volatiles from gerbera (Figure 2A). In none of the replicate experiments did we find a significant preference for the volatiles from leaves with spider mite-damage (χ^2 test, $\alpha = 0.05$). However, when we pooled all data, 63% of the total number of 89 predators made a choice for the leaves with spider mite damage, which is a significant preference (χ^2 test, $0.01 < P < 0.05$).

Subjecting the predators that were reared on spider mite-infested bean leaves to 24 hr of starvation did not increase their response to volatiles from spider mite-infested gerbera leaves (Figure 2B). From the total group of 68 predators, only 60% made a choice for the leaves with spider mite damage, which is not significant (χ^2 test, $\alpha = 0.05$).

The preference for the volatiles from spider mite-damaged gerbera leaves was much stronger for the predators that were reared on gerbera leaves with spider mites (Figure 2C, 2D). When predators were starved for 3 hr (Figure 2C), we found a significant preference for leaves with spider mite damage in all six replicates (χ^2 test, $\alpha = 0.05$). In total, 90% of the predators chose the spider mite-damaged leaves, which is a strong and significant preference (χ^2 test, $P < 0.001$). When predators were starved for 24 hr (Figure 2D), 87% chose the spider mite-infested gerbera leaves (χ^2 test, $P < 0.001$). Pooling the data of both starvation levels shows that the predators that were reared on gerbera with spider mites had a stronger response towards the gerbera volatiles than the predators that were reared on bean with spider mites (2×2 contingency table, $P < 0.001$).

Experiment 3: Influence of Previous Experience on Response of P. persimilis *to Gerbera Volatiles.* As in experiment 2, the predators that were kept for six days on spider mite-infested bean leaves did not have a preference for volatiles from gerbera leaves with spider mite damage (Figure 3). In total, 53% chose the damaged leaves.

Predators that were kept for six days on gerbera showed a preference for damaged gerbera leaves (Figure 3). In two of the three replicates we found a significant preference for the damaged leaves (χ^2 test, $\alpha = 0.05$). In total, 81% chose leaves with spider mite damage. The response towards the spider mite-

TABLE 1. IDENTIFICATION OF CHEMICALS IN HEADSPACE OF UNDAMAGED GERBERA LEAVES AND GERBERA LEAVES INFESTED FOR SEVEN DAYS BY *T. urticae*[a]

Volatile compound	No. in Figure 1	Relative percentage (mean ± SE) in headspace of: Undamaged gerbera leaves	Relative percentage (mean ± SE) in headspace of: Gerbera leaves with spider mite damage
Aldehydes			
Hexanal	9	0.8 ± 0.78 (6*)	0.17 ± 0.141 (0–9)
(*E*)-2-Hexenal	–		0.006 ± 0.0065 (0.7*)
Octanal	18	0.8 ± 0.78 (6*)	0.07 ± 0.061 (0–16)
Nonanal	24	1.2 ± 0.87 (0–0.5)	0.44 ± 0.240 (0–75)
Decanal	29	4 ± 1.8 (0–7)	0.9 ± 0.31 (0–120)
Dodecanal	n.p.	6 ± 5.8 (23*)	
Phenylacetaldehyde	–		0.019 ± 0.0185 (2*)
Unidentified aldehyde	–		0.05 ± 0.032 (0–2)
Alcohols			
1-Butanol	46	27.8 ± 2.26 (4–63)	0.006 ± 0.0065 (0.7*)
2-Butanol	7		0.83 ± 0.200 (6–103)
1-Hexanol	–		0.030 ± 0.0296 (3.2*)
(*Z*)-3-Hexen-1-ol	22	0.25 ± 0.250 (1*)	0.35 ± 0.153 (0–21)
1-Octanol	–		0.025 ± 0.0247 (8*)
1-Nonanol	n.p.		0.10 ± 0.043 (0–13)
1-Dodecanol	n.p.		0.14 ± 0.091 (0–23)
2-Methyl-1-propanol	–		0.0019 ± 0.00185 (0.2*)
2-Phenylethanol	–		0.010 ± 0.0102 (1.1*)
(*E*)-Nerolidol	44		0.20 ± 0.099 (0–34)
Esters			
Ethyl acetate	n.p.		0.09 ± 0.056 (0–21)
Butyl acetate	n.p.	2.2 ± 2.20 (17*)	
Methyl 2-methylbutanoate	n.p.		0.07 ± 0.041 (0–12)
Methyl 3-methyl-2-butenoate	12		0.33 ± 0.120 (2–62)
Hexyl acetate	16	1.7 ± 1.01 (0–4)	0.08 ± 0.058 (0–6.5)
(*Z*)-3-Hexen-1-yl acetate	20	10 ± 4.0 (0–18)	9.5 ± 1.98 (162–557)
(*Z*)-3-Hexen-1-yl 2-methylbutanoate	26		0.07 ± 0.048 (0–3)
(*Z*)-3-Hexen-1-yl 3-methylbutanoate	27		0.26 ± 0.179 (0–19.7)
Unidentified acetates	25		0.28 ± 0.233 (0–25.9)
Ketones			
2-Butanone	2	3 ± 3.2 (25*)	8.3 ± 2.09 (77–511)
3-Buten-2-one	–		0.005 ± 0.0046 (0.5*)
2-Pentanone	4		0.07 ± 0.045 (0–18)
3-Pentanone	–		0.019 ± 0.0185 (2*)
4-Methyl-2-pentanone	n.p.	1.2 ± 1.17 (9*)	
6-Methyl-5-hepten-2-one	21		0.14 ± 0.063 (0–29)

TABLE 1. CONTINUED

Volatile compound	No. in Figure 1	Relative percentage (mean ± SE) in headspace of	
		Undamaged gerbera leaves	Gerbera leaves with spider mite damage
Geranyl acetone	~		0.04 ± 0.040 (4.3*)
Unidentified ketones	11		0.6 ± 0.40 (0–49)
Nitrogen containing compounds			
2-Methyl-2-propenenitrile	5		0.63 ± 0.209 (3–87)
2-Methylpropanenitrile	6		1.32 ± 0.280 (10–136)
2-Methylbutanenitrile	10		0.17 ± 0.057 (0–10)
Unidentified nitriles	8		0.21 ± 0.068 (2–31)
2-Methyl-1-nitropropane	13		0.23 ± 0.056 (1.4–27)
O-Methyl-2-Methylpropanaldoxime	1		0.72 ± 0.235 (7–92)
O-Methyl-2-methylbutanaldoxime	3		1.25 ± 0.294 (0–142)
2-Methylpropanaldoxime	23		0.07 ± 0.032 (0–10)
Aromatic compounds			
Methyl benzoate	~		0.003 ± 0.0032 (0.35*)
Methyl salicylate	n.p.	1.5 ± 1.50 (6*)	0.023 ± 0.0163 (0–2)
2-Methylpropyl-benzoate	~		0.013 ± 0.0130 (1.4*)
n-Butyl benzoate	47	3.9 ± 2.27 (0–3)	0.008 ± 0.0083 (0.9*)
Benzaldehyde	30		3.4 ± 1.44 (0–558)
Terpenoids			
Limonene	n.p.	1.8 ± 1.81 (14*)	0.06 ± 0.038 (0–11)
(*Z*)-*β*-ocimene	14		0.12 ± 0.041 (0.2–18)
(*E*)-*β*-ocimene	15	0.7 ± 0.69 (1*)	4.9 ± 1.12 (43-519)
4,8-Dimethyl-1,3(*Z*),7-nonatriene	17		0.70 ± 0.197 (3–106)
4,8-Dimethyl-1,3(*E*),7-nonatriene	19	26 ± 13.7 (0–17)	32 ± 4.3 (594–2130)
α-Copaene	28		0.53 ± 0.141 (3.7–85)
β-Cubebene	31		0.32 ± 0.159 (0–75)
Linalool	32		1.8 ± 0.72 (9–143)
(*cis*)-*α*-Bergamotene	33		1.1 ± 0.76 (0–87)
(*trans*)-*α*-Bergamotene	34		0.9 ± 0.41 (0.4–170)
β-Elemene	35		2.9 ± 0.77 (8–249)
β-Caryophyllene	36		2.1 ± 0.34 (20–287)
(*E*)-*β*-Farnesene	37		0.38 ± 0.151 (0.3–60)
α-Humulene	38		0.20 ± 0.076 (0.7–30)
Germacrene D	40		1.7 ± 0.48 (8.4–250)
(*E,E*)-*α*-Farnesene	41		3.5 ± 0.57 (16–344)
δ-Cadinene	~		0.016 ± 0.0155 (4*)

TABLE 1. CONTINUED

Volatile compound	No. in Figure 1	Relative percentage (mean ± SE) in headspace of	
		Undamaged gerbera leaves	Gerbera leaves with spider mite damage
Isomer of next compound	42		0.51 ± 0.150 (0–64)
4,8,12-Trimethyl-1,3(*E*), 7(*E*),11-tridecatetraene	43		10.2 ± 1.86 (37–873)
α-Pinene	n.p.	5 ± 5.2 (40*)	
γ-Patchoulene	n.p.	2.5 ± 2.50 (10*)	
Unidentified terpenoids	39		1.0 ± 0.40 (0–184)
Misellaneous			
Indole	45		0.11 ± 0.090 (0.3–30)
Octanoic acid	~		0.04 ± 0.039 (10*)
Unidentified minor peaks		0%	3.3%
Total peak area		13.5–193	1225–8379

[a]Four headspace samples were analyzed for undamaged leaves and five for the leaves with spider mite damage. For each compound, the percentage of the total volatile production is given. Numbers in parentheses represent, for each compound, the absolute minimum and maximum peak area in the individual samples. *Present in detectable levels in one sample only and the peak area for this sample is given. If a compound represents >0.05% of the total peak area, it is indicated with a number in Figure 1 that corresponds with the numbers in this Table. ~ = compound represents <0.05% of total peak area. n.p. = not present in detectable levels in both of the samples that are shown in Figure 1.

induced volatiles of gerbera differed between the two groups of predators (2 × 2 contingency table, $P < 0.01$).

DISCUSSION

It is evident that in gerbera, spider mite damage leads to the production of a large number of volatiles that are not produced by undamaged gerbera leaves. A number of short-chain lipid-derived compounds are present in the headspace of gerbera leaves with spider mite damage. These compounds are usually referred to as green leaf volatiles (Visser et al., 1979) and occur generally in the headspace of artificially damaged leaves or leaves with herbivore damage (see, for example, Dicke et al., 1990a; Turlings et al., 1990; Loughrin et al., 1995; Mattiacci et al., 1995). Two of those compounds, (*Z*)-3-hexen-1-ol and (*Z*)-3-hexen-1-yl acetate, occur in large quantities in the headspace of spider mite-damaged Lima bean leaves. However they do not attract the predatory mite *P. persimilis* when offered as individual compounds in a Y-tube olfactometer (Dicke et al., 1990a).

Less commonly found are the nitrogen-containing compounds such as the nitriles and oximes that are produced by spider mite-damaged gerbera. These

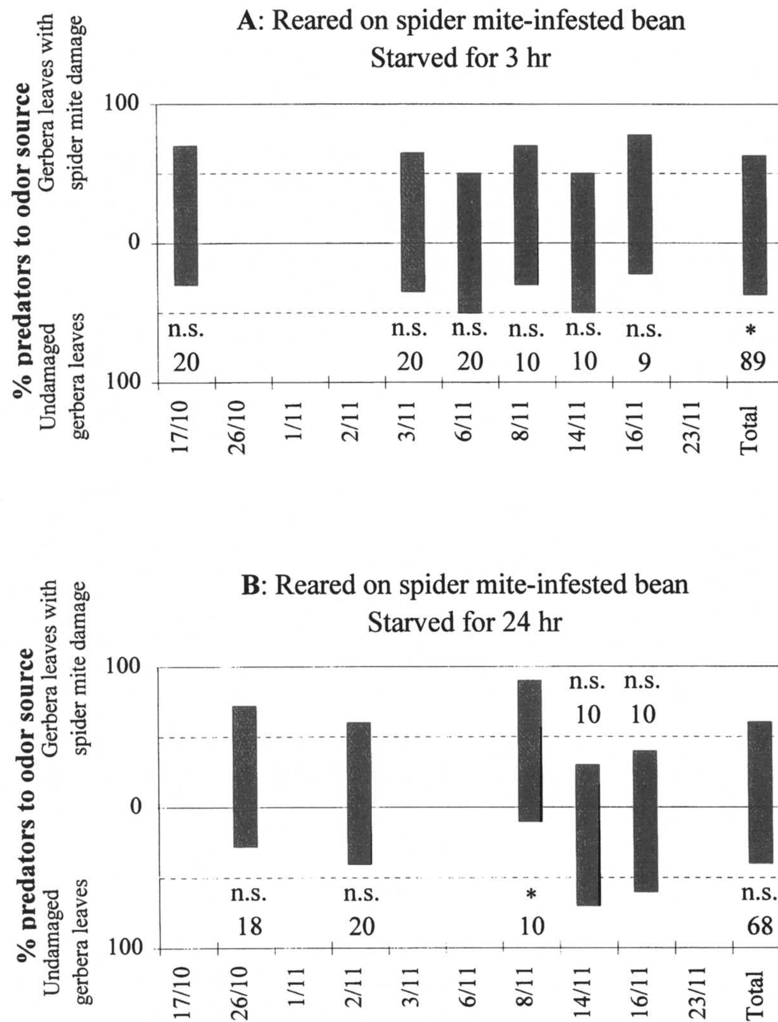
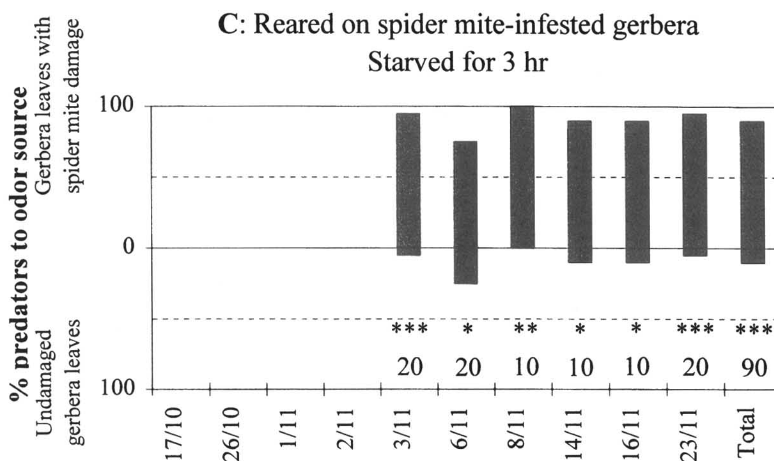
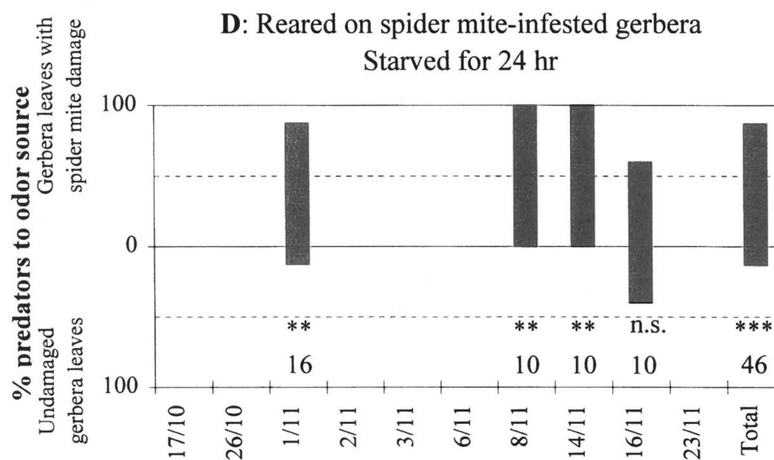


FIG. 2. Response of adult female *P. persimilis* in a Y-tube olfactometer when a choice was given between undamaged gerbera leaves and leaves with spider mite damage. Prior to the experiment *P. persimilis* was either reared on bean with spider mites and starved for 3 hr (A) or for 24 hr (B), or reared on gerbera with spider mites and starved for 3 hr (C) or for 24 hr (D). Dates under the graphs represent the dates on which the experiments





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were performed. Experiments that took place on the same date were performed with the same set of leaves as odor sources. In these cases, predators from each group were tested alternatingly. Data were tested with a χ^2 test; (n.s. = $P > 0.05$, $^{*}0.05 > P > 0.01$, $^{**}0.01 > P > 0.001$, $^{***}P < 0.001$). Numbers below or above bars indicate the number of replicates.

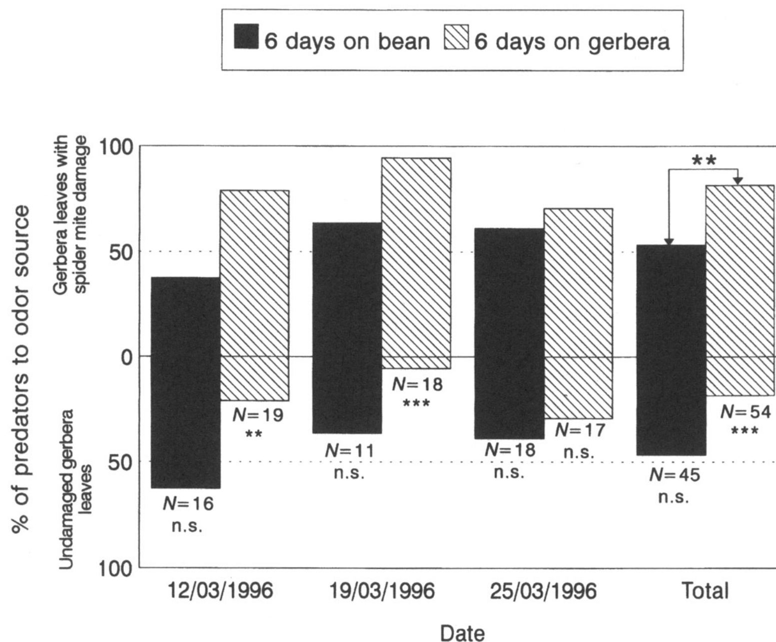

FIG. 3. Response of adult female *P. persimilis* in a Y-tube olfactometer when a choice was given between undamaged gerbera leaves and leaves with spider mite damage. Predators were reared on bean with spider mites and were kept for six days on either spider mite-infested bean leaves or spider mite-infested gerbera leaves. Dates under the graphs represent the dates on which the experiments were performed. Predators from both groups were tested alternately while the same set of leaves was used. Data were tested per bar with a χ^2 test. To test whether the response from the groups of predators differed, we added all numbers for each sub culture separately and performed a 2 × 2 contingency test (n.s. = $P > 0.05$, $^*0.05 > P > 0.01$, $^{**}0.01 > P > 0.001$, $^{***}P < 0.001$).

compounds are also produced by cucumber and apple damaged by *T. urticae*. They were not found in the headspace of artificially damaged cucumber, apple with damage by the spider mite species *Panonychus ulmi*, or *Solanum luteum* and Lima bean with *T. urticae*-damage (Dicke et al., 1990a; Takabayashi et al., 1994b). However, techniques for analysis of headspace volatiles have improved over the last decade and recent analyses of the headspace of spider mite-damaged Lima bean did reveal small amounts of these compounds (Dicke et al., 1999).

Approximately 65% of the volatile blend of spider mite-damaged gerbera consists of terpenoids. Among these, three compounds are present that attract *P. persimilis* when offered as individual compounds in a Y-tube olfactometer. These are the monoterpenes (E)-β-ocimene, linalool, and the homoterpene 4,8-

dimethyl-1,3(*E*),7-nonatriene (Dicke et al., 1990a). Methyl salicylate, which also attracts *P. persimilis*, is present in equally small amounts in the headspace of undamaged leaves and leaves with spider mite damage.

The initial lack of preference of *P. persimilis* for spider mite-damaged gerbera raises the question whether the volatile production is lower than by other plant species. Collection and analysis by Dicke et al. (1999) of an attractive volatile blend of spider mite-damaged bean leaves showed a total volatile production of the same order of magnitude as for gerbera. The production of the attractive compounds (*E*)-β-ocimene and 4,8-dimethyl-1,3(*E*),7-nonatriene are also of the same order of magnitude for bean and gerbera. In contrast, the production of linalool and methyl salicylate is much lower in gerbera, which might explain the initial lack of response of the predators. However, there are many quantitative and qualitative differences between the blends of gerbera and bean, and for most of the gerbera compounds the attractivity to *P. persimilis* is unknown. Hence, it is difficult to know which differences between blends are responsible for the initial difference in effect on *P. persimilis*.

Starvation level obviously does not enhance the response of *P. persimilis* to damaged gerbera leaves, which is in contrast to data for the response to spider mite-damaged pear and apple (Sabelis and Dicke, 1985; Dicke and Sabelis, 1988). Possibly, 24 hr of starvation is not long enough to evoke a response to spider mite-induced volatiles from this host-plant species, as this was found for the response of the predatory mite *Typhlodromus pyri* to volatiles from apple leaves with *T. urticae*. These predators were only attracted to spider mite-damaged apple leaves after 48 hr of starvation (Dicke, 1988b).

Six days of experience in a spider mite patch on gerbera leaves greatly enhanced the response of *P. persimilis*. This is most likely caused by a change in behavior of individual predators rather than a selection for the best-responding individuals. Dispersal of nonresponding predators was not possible during the experiment and, since there was ample food, mortality was not likely to be affected by response to the volatiles.

Takabayashi et al. (1994b) also showed that the response of *P. persimilis* is affected by experience. The predator's response to infested cucumber leaves increased gradually during seven days that it was reared on this plant. It is not known whether this is the result of learning or of physiological changes. For non-host-feeding parasitoid wasp species, which can be kept on alternative food, it is easier to distinguish between these two mechanisms.

Learning by parasitoid wasps has been well studied and documented (see Vet and Groenewold, 1990; Turlings et al., 1993; and Vet et al., 1995 for reviews). Vet and Dicke (1992) suggest that learning to respond to herbivore-induced plant volatiles is expected for a specialist natural enemy of a polyphagous herbivore. Learning is considered to be adaptive if shifts of host-plant species occur frequently over generations, which would prevent the devel-

opment of a fixed response by selection. However, if shifts of host-plant species occur very frequently within generations, learning will have no advantage (Papaj and Prokopy, 1989; Stephens, 1990.

Phytoseiulus persimilis is a specialist natural enemy of a very polyphagous herbivore and thus searches for prey on different host-plant species. Shifts of host plants occur, but not frequently within generations since the predators exterminate a prey patch completely before they disperse (Sabelis and van der Meer, 1986), which generally takes longer than one generation. Hence, for this predator the ability to learn to respond to spider mite-induced volatiles is in accordance with the above mentioned hypotheses (Papaj and Prokopy, 1989; Stephens, 1990; Vet and Dicke, 1992).

In the introduction we formulated a hypothesis regarding the foraging behavior of *P. persimilis*. When we include our present data, the hypothesis changes slightly. Most likely, predators that exterminated a prey patch will try to find a new prey patch by responding to a familiar volatile blend that is associated with the presence of spider mites. Arrival on a host plant species with a very different blend of spider mite-induced volatiles is likely to be a matter of chance. After arrival on such a plant, the response to the spider mite-induced volatiles will likely increase due to experience and possibly over generations due to selection. This will arrest the predators in the prey patch on this new host-plant species.

In contrast to parasitoid wasps in which learning can take place within seconds (Turlings et al., 1993; Vet et al., 1995), the increase in response of *P. persimilis* is a gradual process and is a matter of days rather than seconds (Dicke et al., 1990c; Takabayashi et al., 1994b). This may have consequences for the performance of *P. persimilis* as a biological control agent on certain crops. In commercial mass rearings, *P. persimilis* is commonly reared on bean with spider mites. Introduction of these predators into other crops may result in large losses of predators, since many of them may not be able to find their prey. Previous experience with spider mite-induced volatiles from the crop of interest or the introduction of *P. persimilis* directly on the infested leaves may greatly enhance the effectiveness of the predators as biological control agents.

In conclusion, spider mite damage induces gerbera to produce a large number of volatiles in large quantities. When reared on bean with spider mites, *P. persimilis* is poorly attracted by volatiles from spider mite-damaged gerbera and starvation level hardly influences the response. In contrast, experience with spider mite-infested gerbera leaves greatly increases the response of the predators to spider mite-induced gerbera volatiles.

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IDENTIFICATION AND FIELD EVALUATION OF COMPONENTS OF FEMALE SEX PHEROMONE OF MILLET STEM BORER, *Coniesta ignefusalis*

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Abstract—Five active compounds were detected during analyses of ovipositor washings and effluvia from virgin female *Coniesta ignefusalis* moths by gas chromatography (GC) linked to electroantennographic (EAG) recording from a male moth. These were identified as (*Z*)-7-dodecen-1-ol (*Z*7–12 : OH), (*Z*)-5-decen-1-ol (*Z*5–10 : OH), (*Z*)-7-dodecenal (*Z*7–12 : Ald), (*Z*)-7-dodecenyl acetate (*Z*7–12 : Ac), and (*Z*)-9-tetradecen-1-ol (*Z*9–14 : OH) by comparison of their GC retention times, mass spectra, and EAG activities with those of synthetic standards. Laboratory tests of dispensers for these compounds showed that release rates from polyethylene vials increased to relatively uniform values after three to four days, but release from septa was very rapid and nonuniform and decreased to low levels after two to three days. Trapping tests in Niger showed that the major component, *Z*7–12 : OH, and two of the minor components, *Z*5–10 : OH and *Z*7–12 : Ald, were essential for attraction of male *C. ignefusalis* moths. The most attractive blend contained these three components in a 100 : 5 : 3.3 ratio in a polyethylene vial, which emitted the components in similar proportions to those produced by the female *C. ignefusalis* moth. Water traps baited with this blend containing 1 mg of *Z*7–12 : OH caught more male *C. ignefusalis* moths than traps baited with newly emerged female moths. Addition of up to 10% of the corresponding *E* isomers of the pheromone components had no effect on catches, but addition of the other two minor components detected, *Z*7–12 : Ac and/or *Z*9–14 : OH, to the attractive blend at naturally occurring levels caused significant reductions in trap catch.

Key Words—*Coniesta ignefusalis*, *Acigona ignefusalis*, Lepidoptera, Pyra-

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lidae, sex pheromone, (Z)-7-dodecen-1-ol, (Z)-5-decen-1-ol, (Z)-7-dodecenal, (Z)-7-dodecenyl acetate, (Z)-9-tetradecen-1-ol.

INTRODUCTION

The millet stem borer, *Coniesta* (= *Acigona*) *ignefusalis* Hampson (Lepidoptera: Pyralidae), is an important pest of pearl millet, *Pennisetum glaucum* (L.) R. Br. throughout the West African Sahelian and Soudanian zones (Harris, 1962; N'doye et al., 1984; N'doye and Gahukar, 1987; Youm et al., 1996). During feeding and development, *C. ignefusalis* larvae cause different types of damage, depending upon plant age and the generation. First-generation larvae attack small plants causing "dead heart" and stand loss, whereas second and third generations cause lodging, disruption of the plant vascular system, and inhibition of grain formation due to tunneling (Harris, 1962). In the sub-Saharan African region where pearl millet is the major staple crop grown by subsistence farmers, yield losses due to attack by *C. ignefusalis* range from 15% to total crop failure (Harris, 1962; Ajayi, 1990). In Niger, over 90% of stem borer infestation and damage on millet is caused by *C. ignefusalis* (Youm and Gilstrap, 1993). During research into methods for control of *C. ignefusalis*, the presence of a female sex pheromone was demonstrated in millet fields in Niger (Bako, 1977; ICRISAT, 1989) and seen to be of potential use in an integrated pest management strategy.

This paper reports the results of studies leading to the identification of five compounds produced by virgin female *C. ignefusalis* moths that elicit an antennal response from *C. ignefusalis* male moths and field evaluation of the synthetic compounds as attractants for the male moths.

METHODS AND MATERIALS

Insect Material. Millet stems from the previous season which contained fourth- and fifth-instar diapausing larvae were soaked with water to break diapause and promote further development to pupae. These stems were dissected and pupae dispatched by air from Niger to England where they were sexed, transfered to Perspex containers, and maintained in an environmental cabinet at 32°C, 60% relative humidity during a reversed 12-hr light period and 24°C, 80% relative humidity during the 12-hr dark period.

Pheromone Collection. Ovipositor washings were prepared in hexane from virgin moths 0–2 days old, 7–10 hr into the scotophase, as described by Sower et al. (1973). Effluvia were collected on filters containing activated charcoal (5 mg) by passing charcoal-filtered air (2 liter/ml) over one to three female moths held

in silanized glass containers (12 cm × 4 cm diameter) at 3–12 hr into the scotophase, as previously described (Grob and Zurcher, 1976; Nesbitt et al., 1979; Tumlinson et al., 1982). Trapped volatiles were eluted with dichloromethane (2 × 10 μl).

Release rates of synthetic compounds from dispensers were measured similarly, placing the dispenser in a glass vessel (4 cm × 2 cm diameter) constructed from Quickfit adapters, maintained in a room held at constant temperature (27°C). Volatiles trapped on the charcoal filter were eluted with dichloromethane (3 × 10 μl), pentadecyl acetate (1 μg) added as internal standard, and components were assayed by gas chromatography (GC) as below. Both vials and septa were initially loaded with a mixture containing approximately 0.5 mg each of all five components. Release rates were corrected for the actual amounts of the components in the lures as determined by GC analysis in order to give release rates for all components corresponding to an initial loading of 0.5 mg.

Gas Chromatography. Analyses of natural and synthetic compounds were conducted on a Carlo Erba Mega series 5300 instrument fitted with two Grob split/splitless injectors (200°C) and a flame ionization detector (FID, 240°C). Fused silica capillary columns (25 m × 0.32 mm ID; Chrompack, London, UK) were used, coated with either nonpolar CP Sil5CB (chemically bonded methylsilicone) or polar CP Wax 52CB (chemically bonded Carbowax 20 M equivalent). The carrier gas was helium and inlet pressures of 0.50 and 0.45 kg/cm^2, respectively, were used to maintain a gas velocity of 25 cm/sec in each column. All injections were made in the splitless mode with the split valve closed for 40 sec. Oven temperature for the nonpolar column was held at 60°C for two min, then programmed at 20°C/min to 90°C, then at 1°C/min to 124°C, and then at 4°C/min until the end of the analysis. Conditions for the polar column were the same except that the 1°C/min program was maintained until the end of the analysis.

Electroantennography (EAG). EAG preparations were set up as described in Cork et al. (1990) by using the whole insect and intact antennae and inserting glass microelectrodes filled with saline into the interstitial membranes between the annuli. Coupled GC-EAG analyses were carried out essentially as described by Cork et al. (1990) by splitting the analytical column effluent into two parts, in the present case by using a four-port, zero dead volume connector (Chrompack) instead of push-fit Y tubes. One outlet was attached to the FID and the other to a glass reservoir. Column effluent was pulsed from the reservoir with nitrogen (500 ml/min for 3 sec) at 17-sec intervals over the male *C. ignefusalis* EAG preparation for the duration of the GC analysis.

EAG response profiles from male *C. ignefusalis* to synthetic compounds were recorded essentially as described by Beevor et al. (1986). The test compound was deposited on the inner wall of a glass Pasteur pipet and delivered directly over the antenna in nitrogen (500 ml/min for 3 sec). The interval between successive stimuli was 2 min.

Mass Spectrometry (MS). GC-MS analyses were carried out in electron impact mode on a Finnigan-MAT ITD 700 Ion Trap Detector with open split interface (230°C) to a Carlo Erba Mega series 5300 GC fitted with a fused silica capillary column (25 m × 0.32 mm ID) coated with BP 20 (Carbowax 20 M equivalent; SGE). Carrier gas was helium (0.4 kg/cm^2), splitless injection (200°C), and the GC oven was held at 70°C for 2 min then programmed at 20°C to 120°C, then at 4°C to 230°C.

Synthetic Compounds. Monounsaturated compounds were prepared at NRI by standard acetylenic and Wittig coupling routes followed by argentation chromatography to give material of greater than 99.9% chemical and stereochemical purity.

Field Trials. Field trials of synthetic compounds were carried out in farmers' fields near Sadore, Niger. Initial trials showed water traps to be the most effective trap design for *C. ignefusalis* and were used throughout the studies described here. They consisted of an aluminum tray (28 cm diameter) containing water with a small amount of mineral oil to reduce surface tension, and a plastic lid supported 10 cm above the water surface (Youm et al., 1993). Traps were positioned 0.5 m above ground level (Youm and Beevor, 1995). Pheromone dispensers used in field trials were closed polyethylene vials (22 × 9 × 1.5 mm thick; Just Plastics, London, U.K.), and rubber septa (Aldrich, Gillingham, Dorset, UK; catalog No. Z10,072-2, white) were also evaluated as dispensers in the laboratory. Dispensers were loaded by adding the synthetic compounds and an equal amount by weight of 2,6-di-*tert*-butyl-4-methylphenol (BHT) as antioxidant dissolved in 0.1 ml of petroleum spirit (bp 40–60°C) and allowing the solvent to evaporate. The dispensers were prepared at least two days before use and were mounted in the trap immediately below the lid and above the water surface. In virgin female-baited traps, a single virgin female moth that had emerged in the laboratory from field collected pupae was housed in a metal mesh container (5 × 4 cm) in place of the pheromone dispenser. Female moths were used the first night after emergence and renewed each night.

Traps were positioned approximately 25 m apart in a circular array within a replicate. Moth catches were recorded each day when dispensers were moved clockwise one position within a replicate. Five to seven replicates separated by at least 100 m were carried out for each experiment, and the experiment was run until each treatment had occupied each position once (three to five nights). Mean catches per trap per night in each replicate (x) were transformed to $\log(x + 1)$ to normalize the variance and subjected to analysis of variance (ANOVA). Treatments with zero catches were omitted from the analyses since these have no variance and violate the assumptions of ANOVA. Differences between treatment means were tested for significance by Duncan's multiple-range test (DMRT). Actual mean catches and standard errors are shown in the tables.

RESULTS

Structural Determination. In analyses of ovipositor washings and entrained volatiles from virgin female *C. ignefusalis* moths by GC-EAG, up to five compounds producing EAG activity of double the baseline noise were detected. These were labeled I–V in decreasing order of abundance in entrained volatiles (Table 1), with up to 100 ng of compound I being obtained from a female moth per night by entrainment. GC retention data for the active compounds are given in Table 1 as equivalent chain lengths (ECLs) relative to the retention times of straight-chain acetates (Christie, 1988; Harris and Habgood, 1966). By comparison with ECLs of standard compounds, the differences in ECLs on the two phases (Table 1) suggested that III and V were monounsaturated acetates or aldehydes with 12 carbon atoms, III most probably being an acetate and V with the greater difference in ECLs being an aldehyde. The corresponding differences in ECLs for I, II, and IV were much greater, and these data were characteristic of straight chain, monounsaturated alcohols with 12, 10, and 14 carbon atoms, respectively.

The retention time of each EAG-active compound was compared with those of available isomers on both GC phases. Retention data for component I were consistent only with those for (Z)-7-dodecen-1-ol (Z7–12 : OH) (Figure 1), data for II were consistent only with those for (Z)-5-decen-1-ol (Z5–10 : OH) (Figure 2), data for III were consistent only with those for (Z)-7-dodecenal (Z7–12 : Ald) (Figure 3), data for IV were consistent only with those for (Z)-9-tetradecen-1-ol (Z9–14 : OH) (Figure 4) and data for V were consistent only with those for (Z)-7-dodecen-1-yl acetate (Z7–12 : Ac) (Figure 5) (Nesbitt et al., 1986).

Further evidence for the presence of Z7–12 : Ac in ovipositor washings from

TABLE 1. RELATIVE RETENTION TIMES (ECL) AND ABUNDANCE OF EAG-ACTIVE COMPOUNDS DETECTED IN *C. ignefusalis* OVIPOSITOR WASHINGS AND FEMALE ENTRAINMENT BY LINKED GC-EAG ANALYSIS ON POLAR (CP WAX 52CB) AND NONPOLAR (CP SIL 5CB) COLUMNS

		Relative abundance		Relative retention time (ECL)		
Component	Compound	Washings	Entrainment	CPWax52	CPSil5	Δ[a]
I	Z7–12:OH	100	100	13.16	10.41	2.75
II	Z5–10:OH	2	10	11.16	8.35	2.81
III	Z7–12:Ald	2	6	10.54	9.66	0.88
IV	Z9–14:OH	3	1	15.08	12.67	2.41
V	Z7–12:Ac	1	1	12.31	11.78	0.53

[a](ECL on CPWax52) − (ECL on CPSil5).

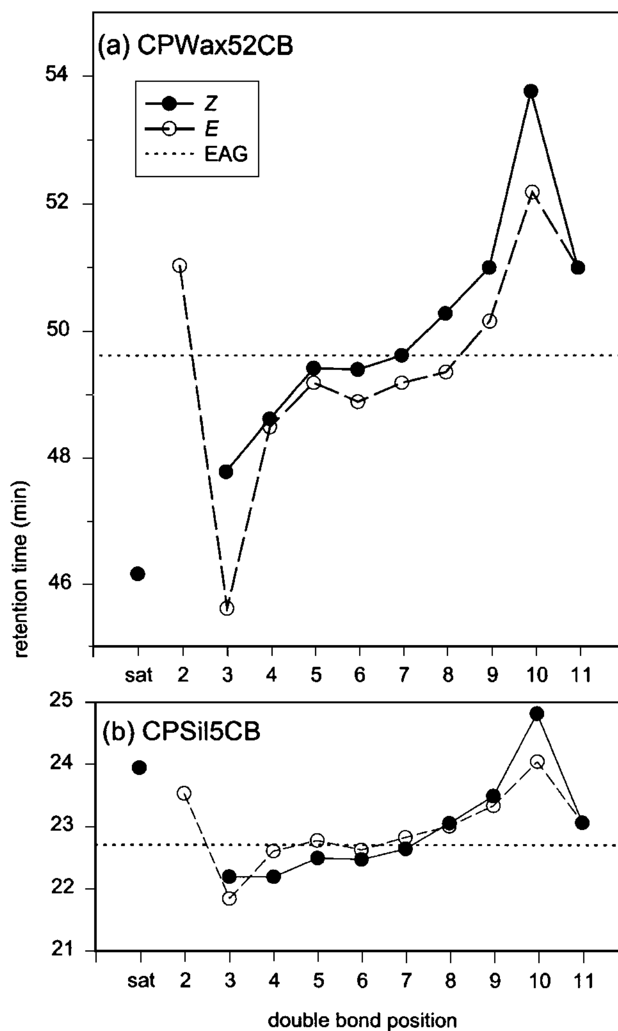

FIG. 1. GC retention times of dodecen-1-ol isomers and EAG-active component I on fused silica capillary columns coated with (a) CP Wax52CB and (b) CP Sil5CB.

female *C. ignefusalis* was obtained by analyzing the washings by GC-EAG using a male *Trichoplusia ni* (Lepidoptera: Noctuidae) moth for the EAG preparation. The main component of the pheromone of this species is Z7–12:Ac (Berger, 1966; Bjostad et al., 1984), and a significant EAG response was obtained at the retention time corresponding to this compound.

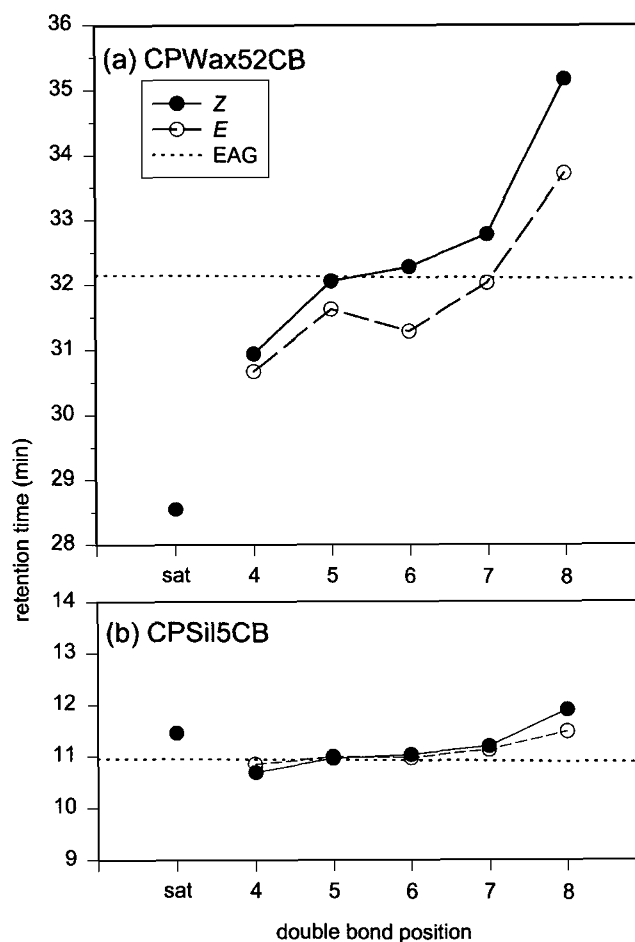

FIG. 2. GC retention times of decen-1-ol isomers and EAG-active component II on fused silica capillary columns coated with (a) CP Wax52CB and (b) CP Sil5CB.

In GC-MS analyses, components with mass spectra matching those of the above five synthetic compounds were recorded at the appropriate retention times, and the presence of dodecan-1-ol at 10–20% of the major component was also established. In particular, the four alcohols all showed a strong ion at m/z 31 (Attygalle et al., 1987).

EAG responses of male *C. ignefusalis* moths to synthetic dodecen-1-ol isomers were recorded by using 5 ng at source off glass. The response of Z7–12 : OH was significantly greater than that to the other isomers tested (Fig-

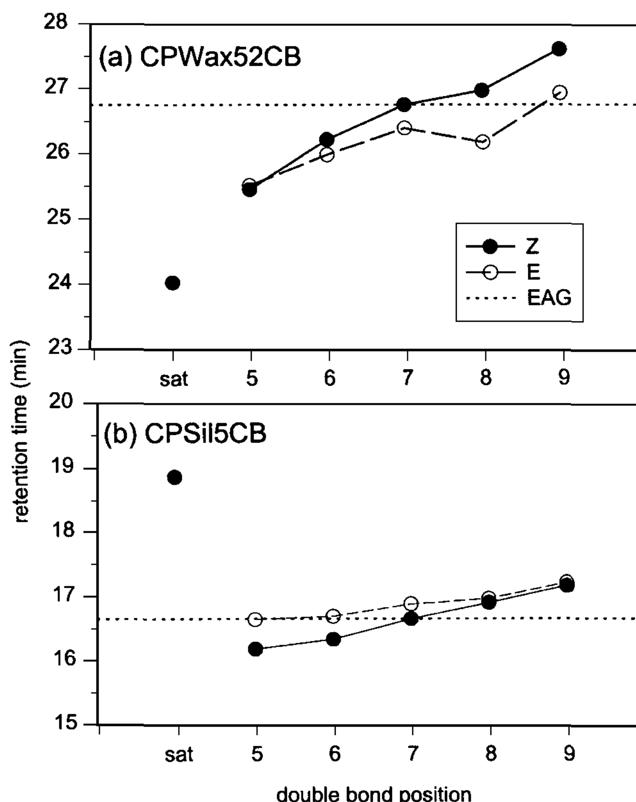

FIG. 3. GC retention times of dodecenal isomers and EAG-active component III on fused silica capillary columns coated with (a) CP Wax52CB and (b) CP Sil5CB.

ure 6). Similarly the EAG response to Z7–12 : Ac was higher than the responses elicited by other (Z)-dodecen-1-yl acetate isomers (Figure 7).

EAG responses of male *C. ignefusalis* to Z5–10 : OH and Z7–10 : OH measured by using linked GC-EAG were 2.61 mV and 0.31 mV above background, respectively, to 4 ng injected (2 ng at the antenna). Similarly, the EAG response to Z7–12 : Ald was 2.72 mV. EAG responses to (Z)-8- and (Z)-9-tetradecen-1-ol at the 5-ng level were similar at 0.42 ± 0.06 mV above blank, but the (Z)-7, (Z)-10, and all four corresponding *E* isomers were inactive at this level.

Pheromone Dispensers. Polyethylene vials and rubber septa were evaluated in the laboratory as dispensers for the five components identified. Results (Figure 8) showed that release was initially rapid from the septa but dropped almost to zero as the contents were exhaused within a few days. The relative ratios of

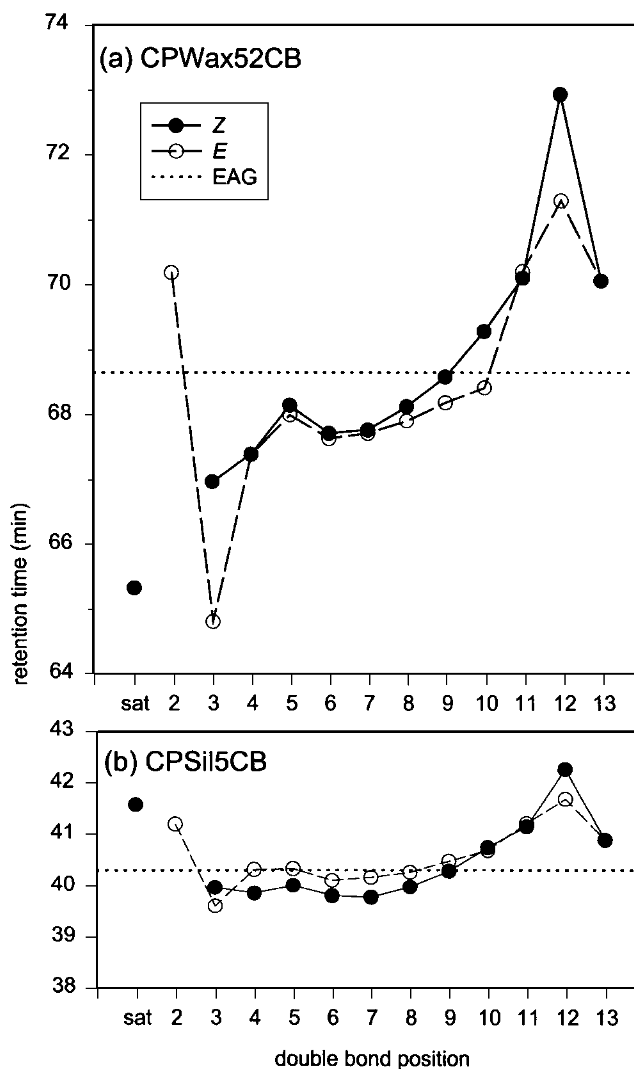

FIG. 4. GC retention times of tetradecen-1-ol isomers and EAG-active component IV on fused silica capillary columns coated with (a) CP Wax52CB and (b) CP Sil5CB.

the different components also changed markedly during this time. In contrast, release rates for all the components from vials increased to reach a fairly constant level after three to four days, and these were maintained for at least the next six days. The release rate of *Z*7–12 : OH for an initial loading of 0.5 mg was 0.72

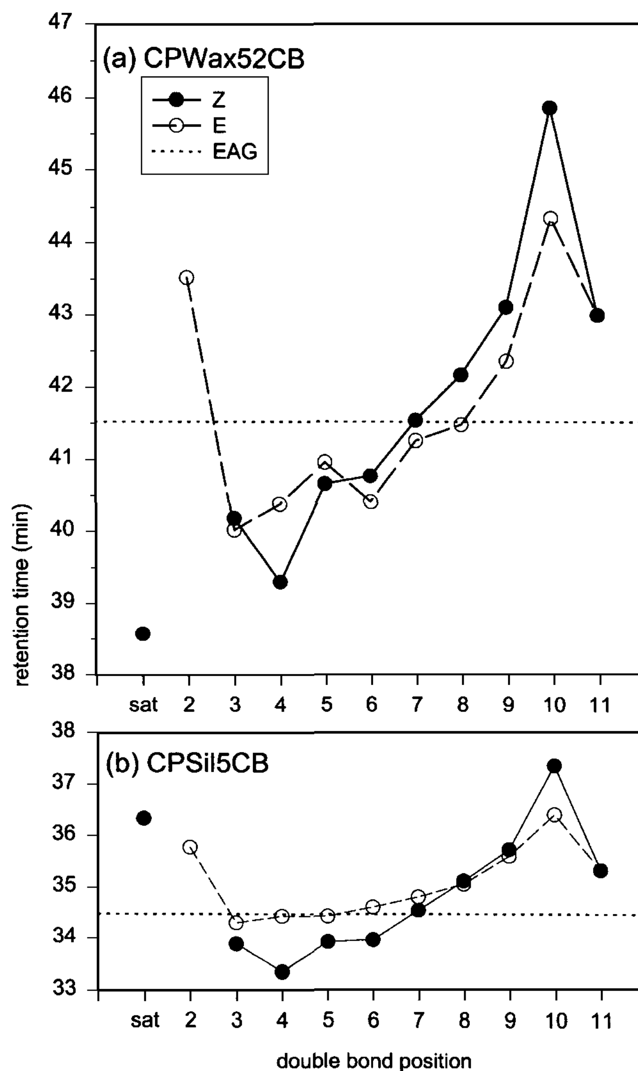

FIG. 5. GC retention times of dodecen-1-yl acetate isomers and EAG-active component V on fused silica capillary columns coated with (a) CP Wax52CB and (b) CP Sil5CB.

μg/hr at 27°C, and the release rates of Z7–12 : Ald, Z5–10 : OH, Z7–12 : Ac, and Z9–14 : OH relative to that of Z7–12 : OH (= 1.00) were 1.83, 1.79, 1.33, and 0.60, respectively.

Field Trials. Preliminary field trials had indicated that the major compo-

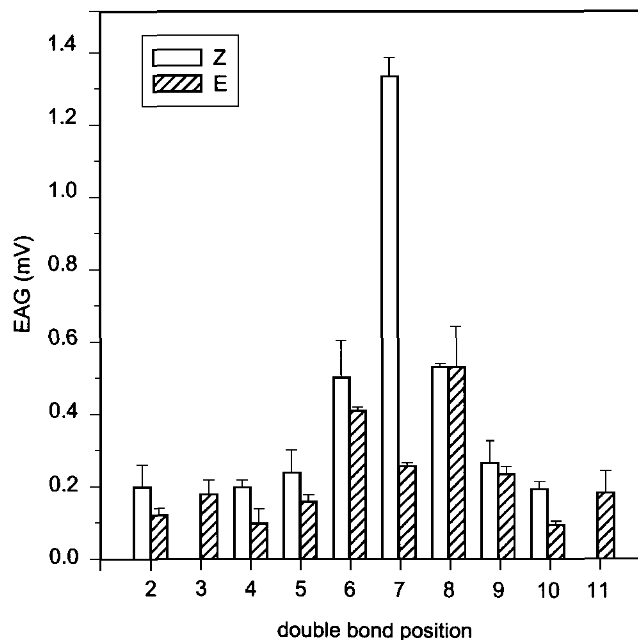

FIG. 6. EAG responses of male *C. ignefusalis* moth to dodecen-1-ol isomers (5 ng off glass; mean of two replicates on each of two insects ±SE).

nent, Z7–12 : OH, was unattractive alone to male *C. ignefusalis* moths but that addition of the minor components Z5–10 : OH and Z7–12 : Ald gave attractive blends. Both minor components were essential for attraction, when using relative amounts of the three components indicated in early analyses of ovipositor washings. (Table 2, experiment 1).

Increasing the amount of Z7–12 : Ald from 5% to 40% with respect to the major component, Z7–12 : OH, showed an optimum level of 5–10% with 20% and 40% significantly reducing catches (Table 2, experiment 2).

Similarly, increasing the amount of Z5–10 : OH from 7.5% to 15% with respect to the major component, Z7–12 : OH, had no effect on catches, but 30% and greater amounts significantly reduced catches (Table 2, experiment 3).

These data suggested that the minor components Z5–10 : OH and Z7–12 : Ald might be optimally combined in the ratio 3 : 2. Results from laboratory determination of release rates from the vials (above) also became available at this time. Together with the amounts of the minor components found in volatiles collected from the female *C. ignefusalis* moth (Table 1), these indicated that Z7–12 : OH, Z5–10 : OH, and Z7–12 : Ald should be present initially in the

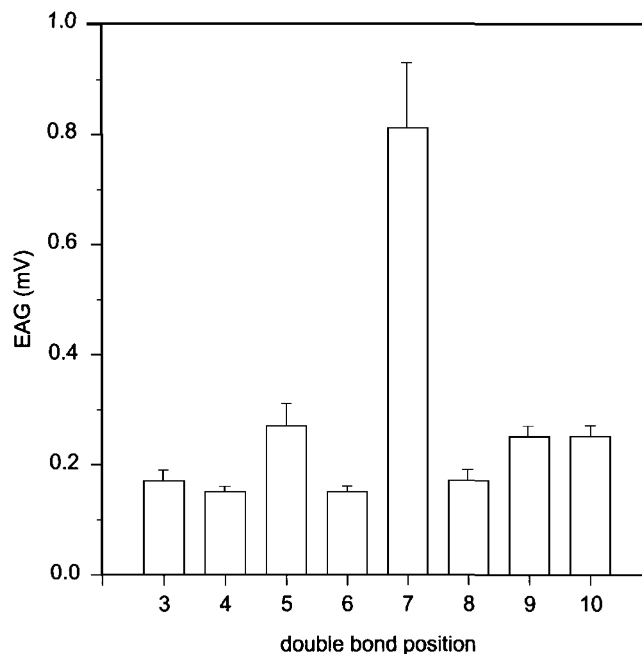

FIG. 7. EAG responses of male *C. ignefusalis* moth to (Z)-dodecen-1-yl acetate isomers (5 ng off glass; mean of two replicates on each of two insects ±SE).

vial at relative amounts of 1000 : 50 : 33, respectively. Further field trials with blends based on these results confirmed that the predicted blend was as attractive as any others tested (Table 3). Increasing or decreasing the relative amounts of the two minor components decreased catches (Table 3).

Addition of either Z7–12 : Ac or Z9–14 : OH to the optimum attractive blend at levels similar to those found in the natural extract and entrained material resulted in significant decreases in catch even at 0.5% and 1.5%, respectively, with respect to the major component (Table 4, experiments 1 and 2). These inhibitory effects seemed to be additive, such that a significant reduction in catches of male moths by the optimum blend was caused by addition of both Z7–12 : Ac and Z9–14 : OH at 0.25% and 0.75%, respectively, with respect to Z7–12 : OH (Table 4, Experiment 3).

Increasing loadings of the optimum pheromone blend in the lures from 0.01 mg to 1.0 mg increased catches of male *C. ignefusalis*, although the difference in catches with 0.1 mg and 1.0 mg was not statistically significant at the 5% level (Table 5, experiment 1).

In a comparison of the attractiveness of the optimum synthetic pheromone

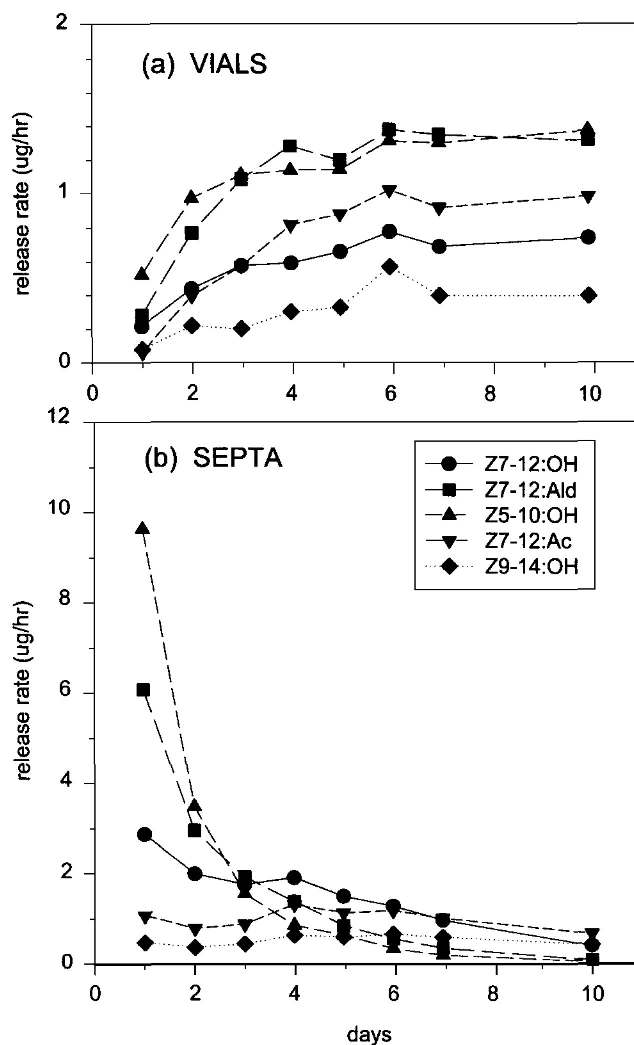

FIG. 8. Release rates of synthetic pheromone components from (a) polyethylene vials and (b) rubber septa at 27°C.

blend and virgin female *C. ignefusalis* moths, water traps baited with the synthetic blend dispensed from a polyethylene vial at a loading of 1 mg Z7–12 : OH caught more than twice as many male *C. ignefusalis* moths as traps baited with a single virgin female moth (Table 5, experiment 2).

The effects of the presence of the corresponding *E* isomers on the attrac-

TABLE 2. CATCHES OF MALE *C. ignefusalis* IN TRAPS BAITED WITH Z7–12:OH AND VARIOUS COMBINATIONS OF Z5–10:OH AND Z7–12:ALD.

Pheromone component (μg)			Mean catch/
Z7–12:OH	Z5–10:OH	Z7–12:Ald	trap/night[a]
Experiment 1 (6 replicates, 5 nights)			
500	75	50	41.2 ± 12.8 a
500	75	—	3.3 ± 0.9 b
500	—	50	0.3 ± 0.2 c
500	—	—	0.5 ± 0.1 c
—	—	—	0.0 c
Experiment 2 (5 replicates, 5 nights)			
500	75	0	1.4 ± 0.8 c
500	75	25	15.1 ± 4.4 a
500	75	50	12.8 ± 3.4 ab
500	75	100	10.1 ± 3.3 b
500	75	200	1.7 ± 0.5 c
Experiment 3 (6 replicates, 5 nights)			
500	0	50	0.3 ± 0.1 c
500	37.5	50	17.1 ± 6.1 a
500	75	50	17.8 ± 5.1 a
500	150	50	4.9 ± 1.7 b
500	300	50	3.8 ± 1.6 b

[a]Actual mean catches ±SE; for ANOVA data transformed to $\log(x + 1)$, means followed by the same letter in each experiment are not significantly different at the 5% confidence level by DMRT.

tiveness of the optimum synthetic pheromone blend were investigated. Addition of up to 10% of the *E* isomers of any of the three pheromone components either singly or together had no consistently significant effects on catches (Table 6). There was even an indication that addition of 2.5% of the *E* isomers increased catches slightly relative to those with the blend of isomerically pure components.

DISCUSSION

This study has shown the presence of five compounds produced and emitted by virgin female *C. ignefusalis*, that elicit an antennal response in male *C. ignefusalis*. These were identified as Z7–12 : OH, Z5–10 : OH Z7–12 : Ald, Z7–12 : Ac, and Z9–14 : OH, and field studies demonstrated that Z7–12 : OH, Z5–10 : OH, and Z7–12 : Ald are essential for attraction of male moths. Polyethylene vials impregnated with a blend of these three components in 100 : 5 : 3.3 ratio emit the components at similar relative rates to those measured from a virgin female moth, and this blend was found to be optimum for attraction of male

TABLE 3. CATCHES OF MALE *C. ignefusalis* IN TRAPS BAITED WITH Z7–12:OH AND VARIOUS AMOUNTS OF Z5–10:OH + Z7–12:ALD IN 3 : 2 RATIO (6 REPLICATES OVER 5 NIGHTS PER EXPERIMENT)

Pheromone component (μg)			Mean catch/
Z7–12:OH	Z5–10:OH	Z7–12:Ald	trap/night[a]
Experiment 1			
500	0	0	0.8 ± 0.1 b
500	6.25	4.16	22.7 ± 5.1 a
500	12.5	8.33	26.1 ± 2.8 a
500	25	16.67	27.2 ± 6.9 a
—	—	—	0.0 c
Experiment 2			
500	25	16.7	27.9 ± 5.4 a
500	50	33.3	19.1 ± 4.3 ab
500	75	50	12.4 ± 3.0 b
500	100	66.7	10.8 ± 1.8 b
500	200	133.3	3.9 ± 1.0 c

[a]Actual mean catches ± SE; for ANOVA data transformed to $\log(x + 1)$, means followed by the same letter in each experiment are not significantly different at the 5% confidence level by DMRT.

moths and at least as attractive as a virgin female moth as measured by catches in baited water traps.

Release rates of the pheromone components from the polyethylene vial dispensers were found to increase over the first three days to reasonably steady levels for at least the next seven days at 27°C. Relative release rates for the two minor components are similar and faster than for the major component, Z7–12 : OH, and hence these components will be depleted more quickly and the relative amounts emitted will decrease over time. However, this will not lead to immediate reductions in catch, and the vials should be effective for at least two weeks in the field. In contrast, rubber septa were shown not to be good dispensers for these relatively volatile pheromone components. As found by Butler and McDonough (1981) and McDonough and Butler (1983), release rates were high and significantly different for the different components such that most of the pheromone was emitted during the first two to three days, and the blend released changed markedly in composition during this period.

Addition of the *E* isomers of the pheromone components to the synthetic blend at up to 10% of the *Z* isomer did not affect catches, so it is not necessary to use materials specially purified to high stereoisomeric purity in lures for *C. ignefusalis*. However, addition of the other minor components shown to be produced by the female moth, Z7–12 : Ac and Z9–14 : OH, markedly reduced catches by

TABLE 4. CATCHES OF MALE *C. ignefusalis* IN TRAPS BAITED WITH ATTRACTIVE BLEND (500 μg Z7–12:OH + 25 μg Z5–10:OH + 16.7 μg Z7–12:ALD) + Z7–12:AC AND/OR Z9–14:OH (6 REPLICATES OVER 5 NIGHTS PER EXPERIMENT)

	Pheromone component (μg)		
Blend	Z7–12:Ac	Z9–14:OH	Mean catch/trap/night[a]
Experiment 1			
542	—	—	8.6 ± 1.3 a
542	1.25	—	7.3 ± 1.3 ab
542	2.5	—	4.1 ± 0.8 b
542	5.0	—	3.8 ± 0.8 b
—	—	—	0.0 ± 0.0 c
Experiment 2			
542	—	—	12.9 ± 2.6 a
542	—	3.75	7.5 ± 1.1 ab
542	—	7.5	6.2 ± 1.2 bc
542	—	15.0	4.5 ± 0.3 c
—	—	—	0.1 ± 0.1 d
Experiment 3			
542	—	—	11.1 ± 2.1 a
542	1.25	3.75	5.5 ± 1.6 b
542	2.5	7.5	3.4 ± 1.5 b
542	5.0	15.0	0.7 ± 0.2 b
—	—	—	0.0 c

[a]Actual mean catches ±SE; for ANOVA data transformed to log(x + 1), means followed by the same letter in each experiment are not significantly different at the 5% confidence level by DMRT.

the three-component attractive blend. When both were present at levels thought to be similar to those produced by the female moth, catches were significantly reduced. Z7–12 : Ac and Z9–14 : OH are potential by-products of the biosynthetic pathways to the components of the attractive blend in the female *C. ignefusalis* moth (e.g., Bjostad et al., 1984) and may act on the same antennal receptors in the male. However, it is not known whether these components have any real behavioral significance within the species and, as they are produced at such low levels, it seems unlikely that they are involved in hindering cross-attraction of other species to help ensure species specificity.

Z7–12 : OH is produced by the female moths in *Autographa gamma* (Dunkelblum et al., 1983), *Eucosma womonana* (Underhill et al., 1987), and *Graphania insignis* (Frérot et al., 1993) and is an essential minor component of the attractive pheromone blends for these species. This compound has also been found in *Actebia fennica* (Struble et al., 1989) *Autographa nigrisigna* (Sugie et al., 1991), *Plusia chalcites* (Dunkelblum et al., 1987), *Cornutiplusia circumflexa*

TABLE 5. CATCHES OF MALE *C. ignefusalis* IN TRAPS BAITED WITH ATTRACTIVE BLEND AT VARIOUS LOADINGS AND IN COMPARISON WITH VIRGIN FEMALE MOTH

Pheromone component (μg)			Mean catch/ trap/night[a]
Z7–12:OH	Z5–10:OH	Z7–12:Ald	
Experiment 1 (6 replicates, 4 nights)			
1000	50	30	41.4 ± 3.8 a
100	5	3	33.4 ± 1.9 a
10	0.5	0.3	14.0 ± 1.8 b
—	—	—	0.0 c
Experiment 2 (7 replicates, 3 nights)			
1000	50	30	57.5 ± 5.6 a
Virgin female moth			23.8 ± 2.8 b
—	—	—	0.2 ± 0.1 c

[a]Actual mean catches ± SE; for ANOVA data transformed to $\log(x + 1)$, means followed by the same letter in each experiment are not significantly different at the 5% confidence level by DMRT.

(Mazor et al., 1991), and *Trichoplusia ni* (Bjostad et al., 1984), although it does not form part of their attractive pheromone blends and in some species actually reduces attraction. Although Z7–12 : OH is produced by female *Agrotis segetum* moths (Löfstedt et al., 1982, 1985, 1986), only Toth and Szõcs (1991) reported it to be attractive to the male moths at high doses in Bulgaria. Z7–12 : OH has also been reported as a component of attractive blends for 30 other species of Lepidoptera, although it has not been shown to be produced naturally in these cases (Arn et al., 1998). To the best of our knowledge, *C. ignefusalis* is the first species recorded in which Z7–12 : OH is produced by the female moth as the major component of the pheromone blend.

Similarly, the minor components Z5–10 : OH and Z7–12 : Ald have been reported previously, but only in *C. ignefusalis* have they been shown to be both produced by the female moths and essential for attraction of the male moths. Thus Z5–10 : OH was identified in the female pheromone gland extracts from several strains of *A. segetum* (Löfstedt et al., 1985, 1986) but was not necessary for attraction of male moths in the field, while field screening showed Z5–10 : OH either alone or in combination with Z5–10 : Ac to be attractive to 16 *Coleophora* species (Priesner et al., 1982; Priesner, 1987; Priesner and Zhang, 1991) and to *Batrachedra pinicolella* (Priesner, 1989). Z7–12 : Ald has previously only been reported as present in *Actebia fennica* (Struble et al., 1989), although it was not necessary for attraction of males in the field, and random field testing has shown blends containing this compound to be attractive to nine other species of Lepidoptera (Arn et al., 1998).

Z7–12 : Ac and Z9–14 : OH have been reported to be produced by many

TABLE 6. CATCHES OF MALE *C. ignefusalis* IN TRAPS BAITED WITH SYNTHETIC PHEROMONE BLEND (500 μg *Z*7–12:OH + 25 μg *Z*5–10:OH + 16.7 μg *Z*7–12:ALD) CONTAINING DIFFERENT AMOUNTS OF CORRESPONDING *E* ISOMERS, (6 REPLICATES OVER 5 NIGHTS PER EXPERIMENT)

Additional pheromone component (μg)			Mean catch/
*E*7–12:OH	*E*5–10:OH	*E*7–12:Ald	trap/night[a]
Experiment 1			
—	—	—	13.5 ± 2.2 b
12.5	—	—	19.3 ± 3.5 a
25	—	—	19.4 ± 3.9 a
50	—	—	17.2 ± 2.4 ab
Unbaited			0.1 ± 0.1 c
Experiment 2			
—	—	—	16.3 ± 1.7 ab
—	0.625	—	20.4 ± 4.3 a
—	1.25	—	17.4 ± 2.5 ab
—	2.5	—	16.8 ± 3.1 b
Unbaited			0.1 ± 0.04 c
Experiment 3			
—	—	—	16.3 ± 3.5 b
—	—	0.42	20.8 ± 3.2 a
—	—	0.84	17.1 ± 3.4 ab
—	—	1.67	21.1 ± 3.7 a
Unbaited			0.1 ± 0.04 c
Experiment 4			
—	—	—	17.4 ± 4.9 b
12.5	0.625	0.42	19.9 ± 3.5 a
25	1.25	0.84	16.2 ± 2.1 ab
50	2.5	1.67	17.1 ± 2.6 ab
Unbaited			0.0 c

[a]Actual mean catches ± SE; for ANOVA data transformed to $\log(x + 1)$, means followed by the same letter in each experiment are not significantly different at the 5% confidence level by DMRT.

species of Lepidoptera, often being an essential component of an attractive pheromone blend (Arn et al., 1998).

Work is in progress to develop pheromone traps for monitoring *C. ignefusalis* in West Africa (Youm et al., 1993; 1997; Youm and Beevor, 1995).

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PHYTOGROWTH PROPERTIES OF LIMONOIDS ISOLATED FROM *Cedrela ciliolata*[1]

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Abstract—An epimeric mixture of the limonoid photogedunin, isolated from the heartwood of *C. ciliolata*, as well as its *R*- and *S*-acetate derivative were evaluated for phytotoxicity in mono- and dicotyledoneous plants. A mixture of epimeric photogedunin **1** and **2**, a mixture of epimeric photogedunin acetate, (*R*)-photogedunin acetate **3**, and (*S*)-photogedunin acetate **4** inhibited seed germination, seedling growth, and root and hypocotyl/coleoptyle growth in all species assayed. The concentration of phytochemicals required for 50% inhibition ranged from 4.5 to 300 μM. Inhibitory plant responses appeared to require that the OH-group at C-23 be acetylated in photogedunin, since the nonacetylated compound showed less phytotoxic activity.

Key Words—*Cedrela ciliolata*, photogedunin, photogedunin acetates, phytogrowth inhibition, seed respiration.

INTRODUCTION

Limonoids are modified triterpenes whose biosynthesis is confined to plants belonging to the order Rutales. The family Meliaceae is known to produce a wide range of these compounds (Champagne et al., 1992). A more limited range of limonoid structures is found in the Rutaceae and Cneoraceae (Champagne et

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al., 1992). Limonoids have a wide range of biological activities, including antifeedant effects and growth-regulating properties on insects, a variety of medicinal effects in animal and humans, as well as antifungal, bactericidal, and antiviral activities (Schmutterer and Ascher, 1984; Jones et al., 1989; Champagne et al., 1992).

As part of our search for biologically active compounds of agrochemical interest, we previously demonstrated that limonoid compounds isolated from the Meliaceae family possess phytotoxic properties (Mata et al., 1996; Lotina-Hennsen et al., 1998). One of them, photogedunin, was isolated for the first time from *Cedrela odorata*, a Jamaican specimen (Burke et al., 1969). Further studies carried out on a Mexican plant, *C. salvadorensis* Standley, yielded a mixture of epimeric photogedunin (Céspedes et al., 1998). This was transformed into two bioactive epimeric photogedunin acetates, 23-(*R*) (**3**) and 23-(*S*) (**4**), which behave as Hill reaction inhibitors on spinach chloroplasts (Céspedes et al., 1998). Whereas the target of 23-(*S*)-photogedunin acetate is localized at the Q_B level (secondary quinone acceptor at the reducing side of photosystem II), 23-(*R*)-photogedunin acetate inhibits PS I electron flow, interacting at the level of b_6f (cytochrome *bf* complex) and at the span from P_{700} to F_x (Céspedes et al., 1998; Lotina-Hennsen et al., 1998).

In this work, we studied *C. ciliolata* S. Watson (Syn. *dugesii*), a small tree that grows on the dry Pacific slope ranging from Jalisco to Chiapas in Mexico through Central America south to the northern area of Panama. We investigated the phytotoxicity of the major nortriterpenoid of this plant: a 23-*R*–23-*S*-mixture of photogedunin, its derivatives, 23-(*R*)-photogedunin acetate, and 23-(*S*)-photogedunin acetate, and its epimeric mixture against two monocots (*Lolium multiflorum* and *Triticum vulgare*) and two dicots (*Trifolium alexandrinum* and *Physalis ixocarpa*). They were assayed for germination, growth, and seed respiration.

METHODS AND MATERIALS

Chemicals and Solvents. All reagents used were either AR or chromatographic grade. Tricine, sorbitol, and MV (methyl viologen) were purchased from Sigma Chemical Co. Methanol, $CHCl_3$, CH_2Cl_2, KCl, $CuSO_4$, NH_4Cl, $MgCl_2$, pyridine, acetic anhydride, silica gel GF_{254} analytical chromatoplates, silica gel grade 60, (70–230, 60 Å) for column chromatography, *n*-hexane, and ethyl acetate were purchased from Merck. Pyridine and acetic anhydride were distilled prior to use.

Apparatus. Electron transfer and oxygen evolution were determined with a Clark type electrode connected to a YSI model 5300 oxygraph.

Plant Material. Young plant material was collected in Morelia, State of

Michoacán, México on February 1997. A voucher sample is on deposit at the ethnobotanical collection of the National Herbarium (MEXU), Instituto de Biología, UNAM. Voucher: M.T. German and P. Tenorio No. 2,174. Register numbers: 800.192 and 800.193.

Isolation and Purification of Photogedunin and Photogedunin Acetates. Gedunin, the epimeric photogedunin mixture (Figure 1), and β-sitosterol were extracted from the heartwood of *C. ciliolata* (1.1 kg) with CH_2Cl_2, followed by chromatography on silica gel (Céspedes et al., 1998). This produced a mixture of compounds (2.91 g, 0.26%). The yield was five times higher than that previously obtained from *C. salvadorensis* plants (Céspedes et al., 1998). Derivatization of the C_{23} hydroxyl group by acetylation with acetic anhydride/pyridine of the 23-*R*–23-*S* photogedunin mixture gave 23-(*R*)-photogedunin acetate and 23-(*S*)-photogedunin acetate (Céspedes et al., 1998).

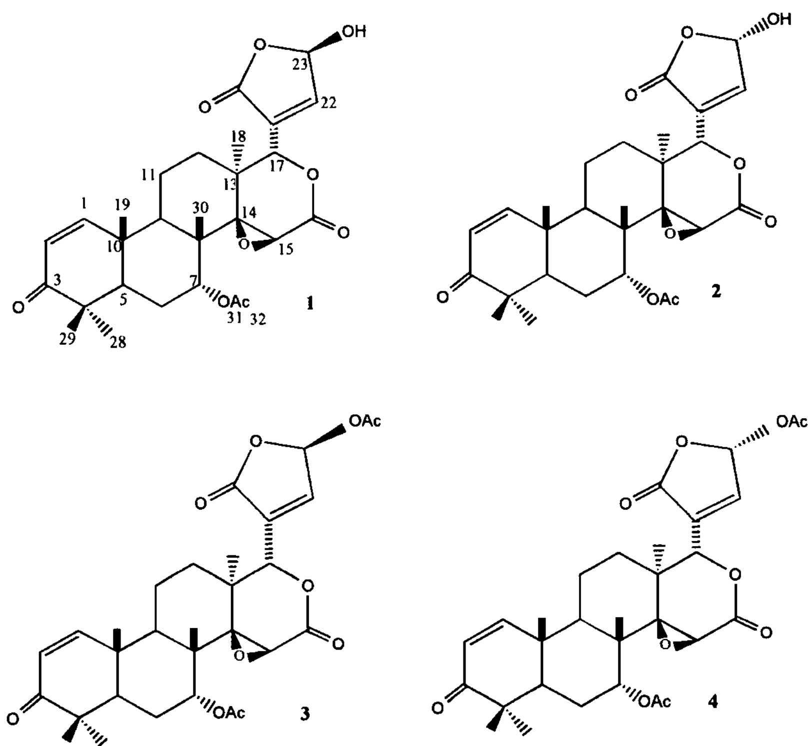

FIG. 1. Structures of 23-(*R*)-photogedunin **1**, 23-(*S*)-photogedunin **2**, 23-(*R*)-photogedunin acetate **3**, and 23-(*S*)-photogedunin acetate **4**.

Seed Germination Bioassays. Lolium multiflorum var. Hercules was purchased from Barenbreig; *Triticum vulgare* var Salamanca, *Trifolium alexandrinum* var. Andino, and *Physalis ixocarpa* were purchased from semillas Berentsen S. A. de C. V. Celaya, Guanajuato, México. For these experiments, 100 seeds of *L. multiflorum*, *T. alexandrinum*, and *P. ixocarpa* were placed on a Petri dish; however, only 40 seeds of *T. vulgare* were required for the assays. The number of seeds used for each experiment was selected so an appreciable change in O_2 uptake could be detected by the oxygraph. Seeds were placed on filter paper (Whatman No. 1) in Petri dishes (85 mm diameter). In three replicate experiments, the paper was wetted with 8 or 10 ml deionized water or test solution (MeOH less than 1%). The dishes were wrapped with Parafilm foil and incubated at 28°C in the dark at intervals of 48 hr. The number of germinated seeds was determined according to the criteria of 1 mm extrusion of the radical. The replication was three for each germination assay. Control seed dishes contained the same amount of seeds, volume of water, and methanol as the test solutions. Seeds were selected for uniformity of size; the damaged ones were discarded (Li et al., 1992).

Growth Bioassays. In two additional replicates, coleoptyle or hypocotyle and root length were measured after 72–96 hr for all seeds germinated.

Bioactivity Guided Isolation of Mixture of Epimeric Photogedunin. Milled heartwood parts of *C. ciliolata* were extracted with CH_2Cl_2, and the initial phytotoxic activity of the resulting extract was evaluated for its growth effects (roots and hypocotyle development) on *Lolium multiflorum* and *Triticum vulgare* seeds and seedlings by using the Petri dish bioassay (Table 1).

The active extract was fractionated by silica gel column chromatography to yield four primary fractions (Fo-1–Fo-4), which were tested for phytogrowth activity. Fraction (F_0-3) contained the epimeric photogedunin mixture,

TABLE 1. PHYTOGROWTH INHIBITORY ACTIVITY OF CH_2Cl_2 EXTRACT, PRIMARY FRACTIONS, ON ROOTS AND SHOOT ELONGATION OF *Lolium multiflorum* AND *Triticum vulgare*

Sample tested	IC_{50} (μg/ml)[a]	
	L. multiflorum	*T. vulgare*
CH_2Cl_2 extract	57.5	62.4
F_0-1	111.4	98.9
F_0-2	75.6	69.9
F_0-3	24.0	23.0
F_0-4	45.1	51.3

[a]IC_{50} = concentration that inhibits 50% growth.

which was analyzed and characterized by R_f, IR, UV, ^1H NMR, and ^{13}C NMR data. This mixture was acetylated and yielded 23-(*R*)- and 23-(*S*)-photogedunin acetates (Céspedes et al., 1998).

RESULTS AND DISCUSSION

Phytotoxicity. Table 1 summarizes the phytotoxic activity of the primary fractions. The mixture of epimeric photogedunin, 23-(*R*)-photogedunin acetate, and 23-(*S*)-photogedunin acetate and the mixture of epimeric photogedunin acetate (Figure 1) were evaluated for their ability to inhibit seed germination of two monocots (*T. vulgare* and *L. multiflorum*) and two dicots (*P. ixocarpa* and *T. alexandrinum*). Growth (roots and coleptyle or hypocotyle development, respectively), dry weight of seedling plants, and seed respiration data were obtained.

Seed Germination. Figure 2 shows the phytotoxic effect of the compounds assayed on seed germination. The mixtures and pure compounds showed similar inhibition profiles. In general, monocot seeds (*T. vulgare* and *L. mutlifloreum*)

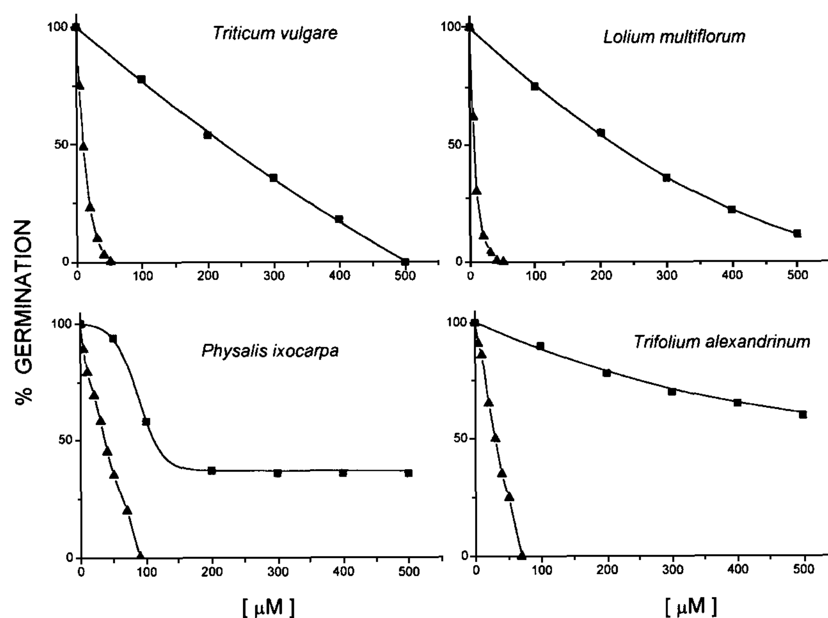

FIG. 2. Effects of solutions of a mixture of photogedunin epimeric (■) and photogedunin epimeric acetates (▲) on germination of *T. vulgare*, *L. multiflorum*, *P. ixocarpa*, and *T. alexandrinum* seeds, expressed as percent of control germination.

TABLE 2. EFFECT OF PHOTOGEDUNIN MIXTURE AND DERIVATIVES ON SEED GERMINATION VALUES

	GI_{50} (μm)[a]			
	Monocot plants		Dicot plants	
Compound	*T. vulgare*	*L. multiflorum*	*P. ixocarpa*	*T. alexandrinum*
Photogedunin mix	220	208	103	[b]
Photogedunin acetate mix	9	6.5	34	29
α-Photogedunin acetate	16	20.7	38.6	32
β-Photogedunin acetate	17.2	10.2	39	35.5

[a]Means of three experiments. GI_{50} = concentration that inhibits 50% of seed germination.
[b]GI_{50} not determinated due to lack of response.

were more sensitive since almost 100% inhibition was observed. The low values of GI_{50} indicate that the mixture of photogedunin acetate is the more powerful inhibitor; values for *T. vulgare* and *L. multiflorum* are 9.0 and 6.5 μM, respectively (Table 2). According to Hatfield and Karlen (1994) and Mohr and Schopfer (1995), preemergence selective inhibitors should be applied after planting but before emergence of weeds or crops, as was done in our tests.

The epimeric mixture of photogedunin has the lowest potency in seed germination; 500 μM was required for 100% inhibition (Figure 2), whereas the other chemicals were 10 times more active (50 μM). These results suggest either that the acetate moiety allows both the mixture and the pure 23-(*R*)- or 23-(*S*)-photogedunin acetates to play a role in inhibition because of lipophilicity or that the hydrophilicity of the free C-23 —OH group of photogedunin (at physiological pH) makes it difficult for photogedunin to reach the target.

Monocot and Dicot Growth. Growth inhibition followed a dose-dependent pattern that stimulated or inhibited germination. Figure 3 shows the inhibitory effect of the photogedunin mixture on root and coleoptyle development of the monocots. The mixture promotes root and hypocotyl development for the dicots. The DI_{50} of the mixture of epimeric photogedunin acetate, 23-(*R*)-photogedunin acetate, and 23-(*S*)-photogedunin acetate were obtained by determining the concentration that induced 50% of growth inhibition of development of roots and shoots (Table 3 and Figure 3). Root development is affected to a larger extent, as indicated by the lowest DI_{50} values, as compared with coleoptyle or hypocotyle development. Growth of dicots was less sensitive to photogedunin acetate, as shown by higher DI_{50} values (Table 3 and Figure 3). The mixture of epimeric

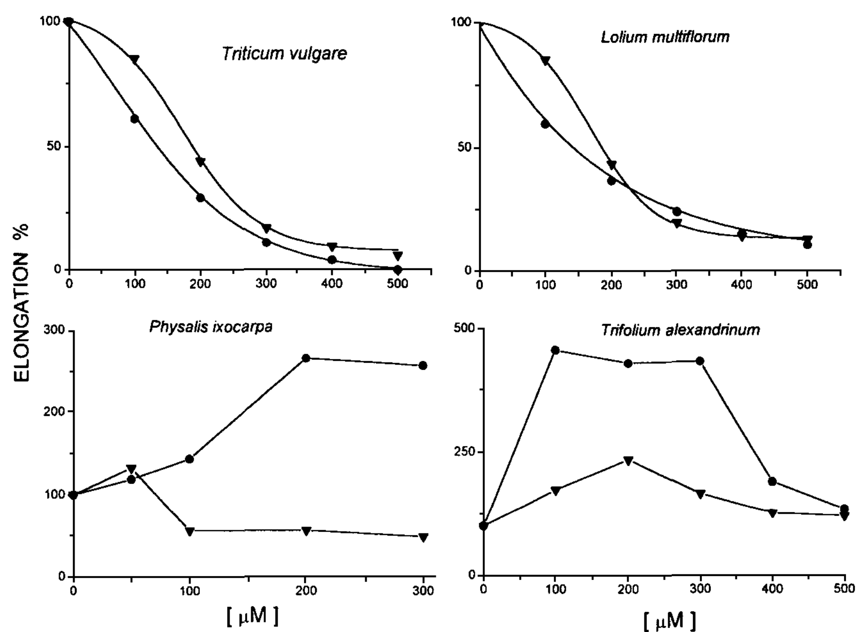

FIG. 3. Effects of solutions of photogedunin mixture on roots (●) and shoots (▼) development of *T. vulgare*, *L. multiflorum*, *P. ixocarpa*, and *T. alexandrinum* seeds, expressed as percent of control.

photogedunin enhances (rather than inhibits) the growth of both *T. alexandrinum* and *P. ixocarpa* roots as concentration increases to 100 and 200 μM. In the case of *P. ixocarpa*, only root development was significantly enhanced at 200 μM (Figure 3). The hypocotyl growth was slightly stimulated at lower concentrations up to 50 μM for *P. ixocarpa*, and to 200 μM for *T. alexandrinum* (Figure 3) and, thereafter, partially inhibited.

As in germination, the mixture of epimeric photogedunin had the lowest inhibitory effects on growth (100% inhibition or lower) at 500 μM for monocots, while for the mixture of epimeric photogedunin acetate 100% inhibition was achieved at 50 and 80 μM for monocots and dicots, respectively (Figure 2). The results indicate that the mechanisms of action of the phytotoxins may be different for growth and germination (Einhellig, 1986).

Dry Weight of Monocotyledonous and Dicotyledonous Plants. Table 4 and Figure 4 show the effect of all compounds on the dry weight of the seedlings. Seedling biomass (dry weight) diminishes with concentration of the assayed compounds in a similar manner to that observed for inhibition of germination. In general, monocots are more susceptible than dicots, which is reflected in their

TABLE 3. EFFECT OF PHOTOGEDUNIN AND DERIVATIVES ON GROWTH OF SEEDLINGS DURING SEED GERMINATION: RDI_{50} AND CDI_{50} VALUES[a]

	Inhibitory conc (μm)							
	Monocot plants				Dicot plants			
	T. vulgare		*L. multiflorum*		*P. ixocarpa*		*T. alexandrinum*	
Compound	Coleoptyle	Root	Coleoptyle	Root	Hypocotyl	Root	Hypocotyl	Root
Photogedunin mix	186	133	185	135	50	200[b]	200[b]	100[b]
Photogedunin acetate mix	24	9.6	16.5	5.4	50	21	28	18
α-Photogedunin acetate	30	28	30.5	19.2	23	17.5	27.8	19
β-Photogedunin acetate	43.8	29	26.9	17.1	30	15.5	25.3	20.7

[a]Means of three experiments. Each value corresponds to the concentration that inhibits 50% of root (RDI_{50}) or coleotyle/hypocotyl (CDI_{50}) development during seedling stage.
[b]Concentration that induces maximum enhancement of growth (root or hypocotyl development).

DWI_{50} values. The growth enhancement activity on *T. alexandrinum* and *P. ixocarpa* is not correlated with dry weight during seedling growth. The diameter of the roots and hypocotyl decreased as a result of adding the tested compounds.

Seeds Respiration During Seed Germination. **The respiratory rate of all**

TABLE 4. EFFECT OF PHOTOGEDUNIN AND DERIVATIVES ON DRY WEIGHT INHIBITION DURING SEEDLING STAGE.

	DWI (μM)[a]			
	Monocot plants		Dicot plants	
Compound	*T. vulgare*	*L. multiflorum*	*P. ixocarpa*	*T. alexandrinum*
Photogedunin mix	66	106	181	138
Photogedunin acetate mix	4.5	6.2	41	7.9
α-Photogedunin acetate	4.6	13	26	22.8
β-Photogedunin acetate	4.6	12.9	26	23

[a]Means of three experiments. Concentration that inhibits 50% of seed germination.

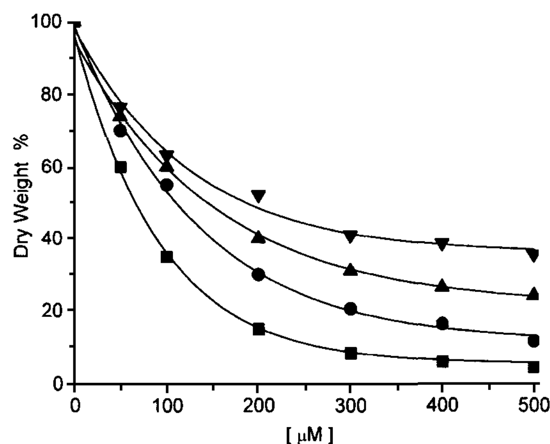

FIG. 4. Inhibition of solutions of mixture of photogedunin epimeric, on dry weight seedling of *T. vulgare* (■), *L. multiflorum* (●), *P. ixocarpa* (▼), and *T. alexandrinum* (▲) seeds, expressed as percent of control.

seeds decreases with concentration of the phytochemicals in a concentration-dependent manner (Table 5 and Figure 5). The only exception is for the photogedunin mixture at 12 μM on *L. multiflorum* seed respiration where enhancement at 12 μM is observed as the time of inhibition increases. However, at higher concentration (70–300 μM), respiration was also inhibited (Figure 5). These results suggest that the photogedunin mixture acts as an uncoupler to phosphorylation at low concentration, but at higher concentrations it either inhibits energy transduction or the respiration redox enzymes. Table 5 shows the RI_{50} values (the concentration of phytochemicals that induce 50% seed respiration inhibition) for all compounds tested. According to their RI_{50} values, *L. multiflorum* seeds are the most sensitive to inhibition. On the other hand, *T. alexandrinum* seeds show the highest resistance to respiration inhibition.

In conclusion, our data indicate that a mixture of epimeric photogedunin is more selective and potent towards monocots than dicots. Respiration processes are involved in the interference action, as these processes were inhibited in a parallel manner by all the mixtures and compounds assayed. Since germination is inhibited with lower doses than respiration, it is possible that these mixtures and compounds have more than one target of interference. When the mixture of epimeric photogedunin is acetylated, the potency of inhibition increases 10 times (Figure 2); thus, we suggest that a natural C-23 derivative is the active one in vivo.

The treatment concentrations for the mixtures of epimeric photogedunin acetate or 23-(*R*)- and 23-(*S*)-photogedunin acetates that reduced seedling

TABLE 5. MEAN CONCENTRATIONS OF PHOTOGEDUNIN AND ACETATE DERIVATIVES THAT INHIBIT 50% OF SEED RESPIRATION[a]

	RI_{50} (μM)[b]			
	Monocot plants		Dicot plants	
Compound	*T. vulgare*	*L. multiflorum*	*P. ixocarpa*	*T. alexandrinum*
Photogedunin	100g	70c	300e	[c]
	200e	185e		
	300b	300d		
Photogedunin	5f, 15e	5c, 7.5e	30g	[c]
acetate mix	10c	10b	50e	40g
	40a	40a		50f
α-Photogedunin	5f	5e	30g	40g
acetate	10e	40a	50e	50f
	30a			
β-Photogedunin	5f	5e	30g	40g
acetate	10e	40a	50e	50f
	30a			

[a]Means of three experiments. Each value corresponds to the concentration that inhibits 50% of seed respiration during germination.
[b]a, value at 8 hr; b, value at 16 hr; c, value at 29 hr; d, value at 18 hr; e, value at 24 hr; f, value at 48 hr; g, value at 72 hr.
[c]RI_{50} was not determined due to lack of seed respiration response to the 24 hr.

growth were low (9–39 μM, Table 2) compared to allelopathic chemicals that have been previously studied under laboratory conditions (Einhelling, 1986). The reported potency of secondary metabolites is in the range of 100–1000 μM for growth reduction by many phenolic acids, around 10 μM for sorgoleone, or at the micromolar level for juglone, which suppresses the growth of several herbaceous species (Einhelling and Souza, 1992; Rietveld, 1983).

Whatever the mechanism(s) of action of the mixture of epimeric photogedunin and its derivatives, they have proved to be good inhibitors of plant growth. They show preemergent phytotoxic properties by inhibiting germination and growth. They also show some degree of selectivity by inhibiting monocotyledoneous species more drastically. Nevertheless, these compounds may also induce postemergent herbicidal effects, since they were found to act as Hill reaction inhibitors (Céspedes et al., 1998).

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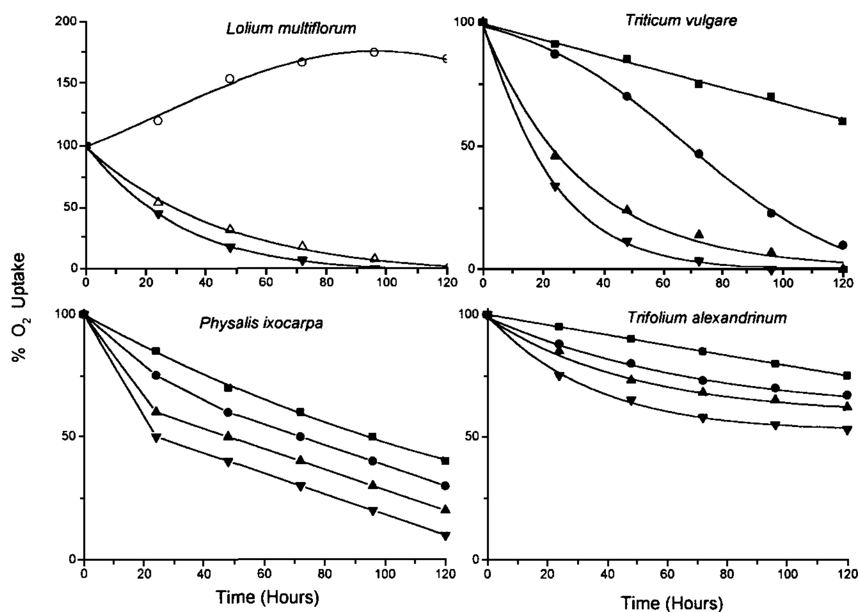

FIG. 5. Inhibition of respiration of *T. vulgare*, *L. multiflorum*, *P. ixocarpa*, and *T. alexandrinum* seeds by solutions of photogedunin mixture, expressed as percent of O_2 uptake rate as a function of control seeds respiration (○), 12 μM, ■, 50 μM; △, 70 μM; ●, 100 μM; ▲, 200 μM; ▼, 300 μM).

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CONDITIONED ALARM BEHAVIOR IN FATHEAD MINNOWS (*Pimephales promelas*) RESULTING FROM ASSOCIATION OF CHEMICAL ALARM PHEROMONE WITH A NONBIOLOGICAL VISUAL STIMULUS

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Abstract—Fathead minnows (*Pimephales promelas*) adopt antipredator (alarm) behavior when they detect alarm pheromone released from an injured conspecific. This is an adaptive response since alarm pheromone is generally released only in the context of a predation event. Alarm reactions may also occur in response to chemical and visual stimuli that minnows learn to associate with release of alarm pheromone. Here, we tested if fathead minnows can learn to associate a nonbiological, visual stimulus with predation risk. Minnows were simultaneously exposed to red light and conspecific alarm pheromone, inducing an alarm reaction. When retested using red light alone, small shoals of minnows displayed an antipredator response: dashing movements and disorganized swimming followed by decreased height in the water column and increased shoal cohesion. This resulted from a single-trial exposure to the combined cues and demonstrates a robust ecological mechanism by which minnows learn to recognize indicators of predation risk that may vary in space and time. However, learning to associate risk with biologically irrelevant stimuli may be an ecological liability. How minnows discern between relevant and irrelevant stimuli in nature is not known.

Key Words—Fathead minnow, *Pimephales promelas*, alarm pheromone, Schreckstoff, learned recognition of predation risk, red light.

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INTRODUCTION

The fathead minnow (*Pimephales promelas*), like other members of the superorder Ostariophysi, possesses specialized epidermal club cells that contain alarm pheromone (Frisch, 1941; Pfeiffer, 1977; Smith, 1992; Chivers and Smith, 1998). When minnows are injured by a predator, these epidermal cells rupture, releasing alarm pheromone (*Schreckstoff*) into the water. This compound, reputedly hypoxanthine-3-(*N*)-oxide (Pfeiffer et al., 1985), serves to alert conspecifics to predation threat (Pfeiffer, 1963) and invokes a range of species-specific antipredator behaviors such as increased shoaling, seeking refuge, dashing, freezing, and area avoidance (Magurran, 1989; Mathis and Smith, 1992; Chivers and Smith, 1994a,b; Wisenden et al., 1995). Since ostariophysan fishes account for 28% of all known fish species and 64% of all freshwater species (Nelson, 1994), the *Schreckstoff* reaction system is extremely common and of great ecological importance.

Alarm pheromone release facilitates predator recognition in minnows (Magurran, 1989; Suboski et al., 1990). For instance, Göz (1941) found that blinded European minnows (*Phoxinus phoxinus*) displayed no initial response to pike (*Esox lucius*) odor. After being attacked, and thus exposed to pike odor and alarm pheromone simultaneously, the minnows reacted with alarm to pike odor alone. This paradigm for associated learning of novel chemical cues has subsequently been demonstrated for a number of species: fathead minnows (Mathis and Smith, 1993; Chivers and Smith, 1995), European minnows (Magurran, 1989), brook stickleback (Chivers et al., 1994), and damselfly larvae (Chivers et al., 1996; Wisenden et al., 1997). Only four previous fish studies have documented learned recognition of a visual stimulus by association with injury-released alarm hormone (Suboski et al., 1990; Chivers and Smith, 1994b; Hall and Suboski, 1995; Brown et al., 1997).

Although visual learning has been documented in fathead minnows in conjunction with the sight of pike (Chivers and Smith, 1994b; Brown et al., 1997) it has been suggested that minnows naive to the sight of pike may be genetically predisposed to avoid large fish as a consequence of previous generations of minnows occurring in similar habitats as pike. To determine if learning can occur in response to visual cues to which minnows cannot be genetically predisposed, a biologically irrelevant stimulus must be presented. The objective of this study was to determine if fathead minnows can learn to associate a nonbiological stimulus (a red light) with predation risk.

Depth and duration of vertical movement in the water column are reliable indicators of alarm in minnow species (Hall and Suboski, 1995; Chivers and Smith, 1998). These criteria were used to indicate alarm behavior in small groups of male fathead minnows exposed to alarm pheromone or red light stimulus alone. Both stimuli were applied simultaneously, and the response to light

stimulus alone was subsequently evaluated. We predicted that if fathead minnows can learn to associate a nonbiological visual stimulus with predation risk, the second application of red light alone should induce an antipredator response.

METHODS AND MATERIALS

Minnows. Adult male fathead minnows, 4–5 cm in length, were obtained from Hasse Lake, Alberta (53°20′N, 115°5′W) and maintained in a 300-liter flow-through aquarium at 15°C on a 16L : 8D photoperiod for at least two weeks prior to use. While in the holding tank, minnows were fed to satiation daily with Nutrafin fish flakes and frozen brine shrimp (*Artemia salina*). Feeding was restricted to the holding aquarium, so as to avoid the possibility that food odors might mask alarm pheromone released into the testing aquaria.

Apparatus. All experiments were conducted in aquaria with dimensions 40 × 19 × 19 cm. Each aquarium had three opaque sides painted light blue, a 1-cm layer of naturally colored gravel on the bottom, and was filled with 14.5 liters of dechlorinated, 21°C water. A single aeration stone and a 1-mm-diameter polyethylene tube (for the introduction of alarm pheromone) centered both horizontally and vertically were affixed to the back wall of each tank. Horizontal lines on the front wall of the aquarium divided the water column into four equal layers. The visual stimulus was a 9-V bulb covered by a transparent red filter, positioned external to and centered against the clear front pane of the aquarium. When used as a stimulus, the light was illuminated for 3 min (Hall and Suboski, 1995).

To collect alarm pheromone, two female minnows were killed via cervical dislocation. A scalpel was used to make 10 superficial epidermal cuts on each side of the fish. Alarm pheromone was extracted from females, rather than male minnows because the males were either sexually mature or only recently sexually regressed. Sexually mature male fathead minnows do not produce alarm pheromone (Smith, 1976). Ten milliliters of distilled deionized water (DDW) was washed over each fish. One-milliliter aliquots of this solution were kept frozen at −20°C until use. Aliquots were thawed as needed and diluted in 9 ml DDW.

Alarm pheromone exposure consisted of the addition of 5 ml of dilute extract solution. As a control, 5-ml samples of DDW not containing alarm pheromone were frozen and stored until needed. Introduction of water or alarm pheromone, light operation, and behavioral observation were performed from behind a black fabric blind. The pheromone solution injected into the treatment aquaria appeared clear; therefore, any behavioral response was likely not due to visual detection of a cloud of pheromone solution.

Procedure. Six minnows were used in each treatment group, following the

methods used by Hall and Suboski (1995). Six male minnows were subjected to five sequential experiments conducted in a single test session: (1) administration of water not containing alarm pheromone, (2) administration of alarm pheromone, (3) administration of a 3-min light stimulus without alarm substance, (4) administration of a light stimulus in conjunction with alarm substance, and (5) administration of light stimulus without alarm substance. The experimental series was repeated a few days later with a second group of six male minnows. The same minnows were not used in both sessions.

Six test minnows were transferred from a large holding tank to a test aquarium and left undisturbed for 24 hr. For each subsequent test (1–5 above), test fish were transferred to a new test aquarium containing fresh dechlorinated water. Transfers of fish between aquaria were conducted carefully so as not to release alarm pheromone or transport it from one tank to another. All six members of each group were placed in a 300-ml water-filled beaker to be rinsed free of any pheromone before being placed into the treatment aquarium. This was done by pouring off much of the water from the beaker and replacing it with fresh dechlorinated water. This procedure was repeated three times, after which the fish were released into the aquarium (Hall and Suboski, 1995). Curtains, lights, and remote administration tubes were prepared prior to transferring the minnows to the testing aquaria.

The schedule of pre- and poststimulus observations were based on that used by Suboski et al. (1990). Baseline observations were made at time zero (stimulus presentation) minus 9, 6.25, 4, 2.25, 1, and 0.25 min and at 0 min. Poststimulus observations were made at time zero plus 0.25, 1, 2.25, 4, 6.25, 9, 12.25, 16, 20.25, 25, 36, 49, 64, and 81 min.

Scoring. For all treatments, scan sampling performed by a single observer determined the vertical location of individual fish at each observation period. Each fish was assigned a score corresponding to its vertical location. A fish in the lowest level (level 1) was assigned a score of 0. Fish located within layers 2, 3, and 4 (layer 4 is the top) were assigned scores of 0.5, 1.5, and 2.5, respectively. In this manner, the mean score of the six fish at each time interval was determined. The means for each of the seven prestimulus intervals were averaged to give a single baseline mean. This baseline mean was used to convert the mean score of each of the 14 poststimulus time interval means into an index of vertical distribution. Each poststimulus observation mean was divided by the sum of itself plus the baseline mean. The resulting vertical distribution index could thus range from zero (all six fish in the lowest level) to 0.5 (the poststimulus mean is equal to the baseline mean) to 1.0 (the poststimulus mean is greater than baseline). In this type of design, the group of fish, instead of the individual fish, is the experimental unit.

Qualitative observations regarding individual fish posture, shoaling, swimming, and rate of fin flicking were also made at each observation period.

Alarm Status and Analysis. A group of fish was considered alarmed if the first six of the 14 poststimulus means were below baseline (Hall and Suboski, 1995). Binomial statistical tests were used to determine the P value for the number of observations below the prestimulus mean for each treatment (Hall and Suboski, 1995). The probability of six sequential vertical indices randomly occurring below the prestimulus mean is $P = 0.0078$ (binomial test).

RESULTS

Treatment 1—Water with No Alarm Substance. Injection of DDW via the 1-mm-diameter polyethylene tube resulted in no observable change in vertical distribution of either test group (binomial test, group 1 $P = 0.4240$, group 2 $P = 1.0000$, binomial test; Figure 1). No changes in the distribution or behavior of the minnows was observed. The result of this control treatment eliminates the possibility that water currents or temperature changes caused by injection of a solution resulted in any behavioral response in the minnows.

Treatment 2—Alarm Substance Alone. Injection of alarm substance provoked an alarm response. For both groups, injection of alarm substance resulted in vertical distributions lower than the pretreatment mean for all 14 of the post-treatment observation periods (binomial test, both groups $P = 0.0001$; Figure 2). Alarm substance was, thus, effective in inducing a strong alarm response.

Administration of alarm substance corresponded with an increase in short, rapid swimming or darting motions followed by increased shoaling, increased frequency and rapidity of pectoral fin motion, and cessation of substrate probing.

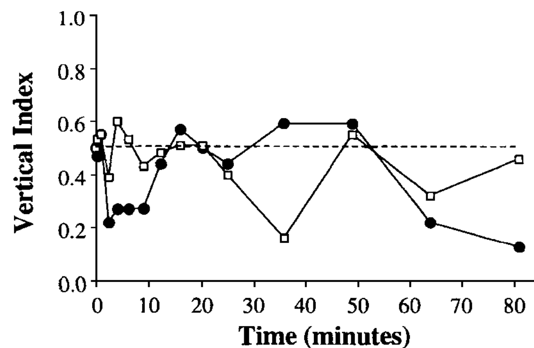

FIG. 1. Vertical distribution of a shoal of six fathead minnows over 81 min following the introduction of distilled deionized water (at time zero). Vertical distributions are relative to the baseline mean established during the 9 min prior to stimulation. Each line represents a replicate experiment. Group 1, circles; group 2, squares.

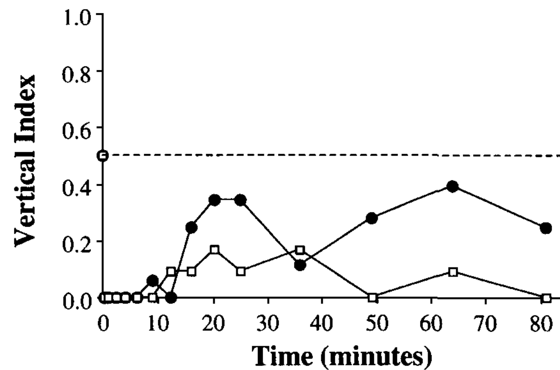

FIG. 2. Vertical distribution of a shoal of six fathead minnows over 81 min following the introduction of distilled deionized water containing alarm pheromone (at time zero). Vertical distributions are relative to the baseline mean established during the 9 min prior to stimulation. Each line represents a replicate experiment. Group 1, circles; group 2, squares.

Treatment 3—Light Stimulus Alone. This treatment was necessary to demonstrate that red light was initially an irrelevant stimulus that would not induce alarm behavior. Neither group displayed alarm behavior in response to the light (binomial test, group 1 $P = 0.1796$, group 2 $P = 1.0000$; Figure 3). Initially, minnows congregated near the light in loose shoal formation for the first

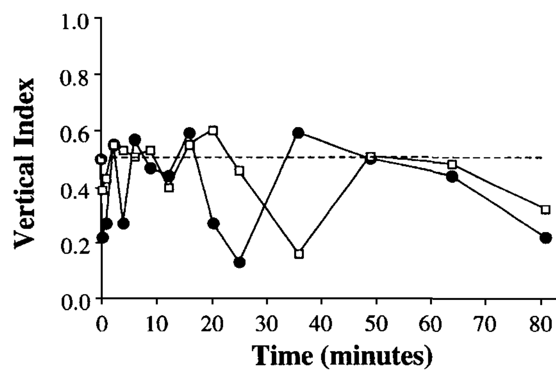

FIG. 3. Vertical distribution of a shoal of six fathead minnows over 81 min following illumination of a red light for 3 min (at time zero). Vertical distributions are relative to the baseline mean established during the 9 min prior to stimulation. Each line represents a replicate experiment. Group 1, circles; group 2, squares.

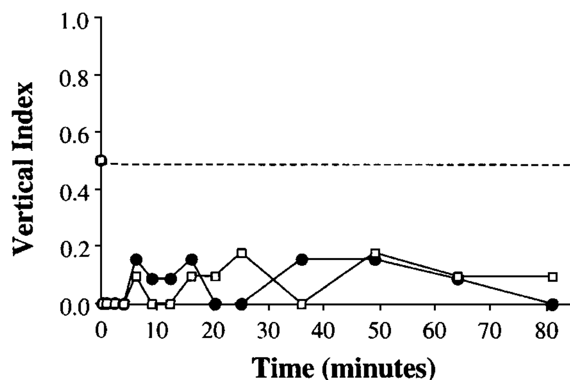

FIG. 4. Vertical distribution of a shoal of six fathead minnows over 81 min following introduction of distilled deionized water containing alarm pheromone plus illumination of a red light for 3 min (at time zero). Vertical distributions are relative to the baseline mean established during the 9 min prior to stimulation. Each line represents a replicate experiment. Group 1, circles; group 2, squares.

three observation periods before dispersing to a more even distribution. The light however, remained illuminated for the first six observation periods, indicating that after the initial visual inspection, the minnows perceived no threat from the novel stimulus.

Treatment 4—Alarm Substance and Light Stimulus. This treatment was required to demonstrate that alarm behavior occurred at the time of conditioning. As with treatment 2, alarm behavior was noted in both aquaria (binomial test, both groups $P = 0.0001$; Figure 4). All four qualitative behaviors in response to alarm pheromone alone occurred in response to the combined stimulus of red light plus alarm pheromone.

Treatment 5—Light Stimulus Alone. This treatment tested if a single tandem exposure to red light and alarm pheromone resulted in learned recognition of red light as an indicator of predation risk. Group 1 showed vertical indices below the prestimulus mean for all observation periods (binomial test, $P = 0.0001$; Figure 5). Group 2 displayed alarm behavior for the first eight observation periods (binomial test, $P = 0.0129$; Figure 5). This red light treatment resulted in the same spatial organization of minnows as when pheromone was added (treatments 2 and 4), except the shoaling response was delayed.

As with treatments 2 and 4, increased rates of fin flicking and increased shoal cohesion were observed. However, the intensity and duration of these behaviors were less than the responses in treatments 2 and 4. In addition, no minnows were observed to cross in front of the light while it was illuminated.

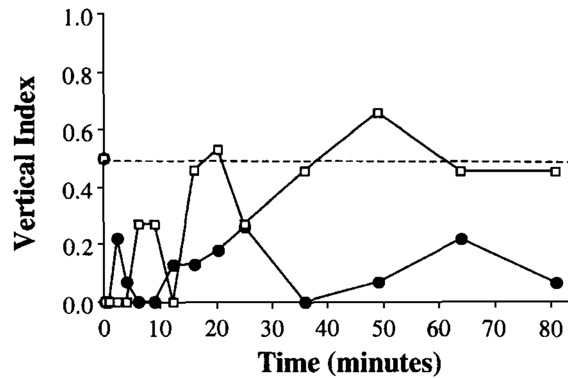

FIG. 5. Vertical distribution of a shoal of six alarm pheromone plus red light preconditioned fathead minnows over 81 min following illumination of a red light for 3 min (at time zero). Vertical distributions are relative to the baseline mean established during the 9 min prior to stimulation. Each line represents a replicate experiment. Group 1, circles; group 2, squares.

DISCUSSION

This study shows that a single simultaneous exposure to alarm pheromone and a novel stimulus conditions an alarm response in fathead minnows (Chivers and Smith, 1998). This is the first demonstration of a learned response to nonbiological visual stimulus in fathead minnows and an important confirmation of a similar finding in zebra danios, *Brachydanio rerio* (Suboski et al., 1990; Hall and Suboski, 1995).

This study and others (Suboski et al., 1990; Chivers and Smith, 1994b, 1995; Hall and Suboski, 1995) underscore the flexibility of this type of learning. The learning mechanism confers upon individual minnows the ability to learn to recognize indicators of predation risk across a broad range of spatial and temporal scales where predator diversity and abundance can be expected to vary widely. This occurs after a single simultaneous encounter with alarm substance and a novel chemical or visual stimulus. Although it is possible to invoke a number of scenarios where this learning paradigm would be maintained by natural selection, if minnows can be easily tricked into learning to respond to nonbiological or irrelevant stimuli, then this learning paradigm may represent a fitness liability. In nature, a response to irrelevant stimuli would carry significant cost. This leads to the intriguing question of how minnows assign a hierarchy of salience to visual or chemical stimuli that may be present at the time of alarm pheromone release.

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VARIATION IN ALLYL ISOTHIOCYANATE PRODUCTION WITHIN *Brassica* SPECIES AND CORRELATION WITH FUNGICIDAL ACTIVITY

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Abstract—*Brassica nigra* (black mustard) and *B. juncea* (Indian mustard) genotypes were tested for pathogen suppression and release of allyl isothiocyanate (AITC), a fungitoxic volatile produced in mustard tissue after enzymatic hydrolysis of allyl glucosinolate (sinigrin). In bioassays, 28 genotypes of *B. nigra* and 35 genotypes of *B. juncea* were screened for inhibition of the potato pathogens *Helminthosporium solani* and *Verticillium dahliae* by volatiles released from macerated leaf tissue. Release of AITC from plant tissue was quantified by gas chromatography; isothiocyanate profiles were determined by headspace analysis. All mustard genotypes produced compounds that suppressed radial growth of both fungi. Growth suppression and AITC release differed significantly ($P < 0.001$) among genotypes of *B. nigra* and *B. juncea*. Mustard treatments releasing >1.2 mg AITC/g plant tissue were fungicidal to both pathogens. Headspace analysis confirmed that allyl glucosinolate was the major glucosinolate in all genotypes of *B. nigra* tested; most genotypes also produced 2-phenylethyl-isothiocyanate (ITC). *Brassica juncea* genotypes produced variable amounts of AITC and other volatiles with antimicrobial activity, including 2-phenylethyl-ITC, benzyl-ITC, and 3-butenyl-ITC. Evaluating mustards from geographically diverse locations allowed selection of mustard genotypes that may be useful in breeding programs designed to develop disease-suppressing green manure cultivars.

Key Words—Mustard, *Brassica juncea*, *Brassica nigra*, fungitoxic, allyl isothiocyanate, Brassicaceae, glucosinolate, 2-phenylethyl-ITC, *Helminthosporium solani*, *Verticillium dahliae*.

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INTRODUCTION

Members of the Brassicaceae can inhibit fungal plant pathogens when grown as a green manure crop or added to soil as seed meal or plant residues. White mustard [*Brassica hirta* (L.) Moench], black mustard [*B. nigra* (L.) W. Koch], cabbage (*B. oleracea* L.), and rapeseed (*B. napus* L.) reduced population densities of *Aphanomyces euteiches* Drechs. or other root rot-causing pathogens of peas (Papavizas, 1966; Lewis and Papavizas, 1971; Muehlchen, 1990; Williams-Woodward et al., 1997). The wilt pathogens *Fusarium oxysporum* Schlecht.: Fr. f. sp. *conglutinans* and *Verticillium dahliae* Kleb. were inhibited by various crucifer amendments (Ramirez-Villapudua and Munnecke, 1988; Subbarao and Hubbard, 1996). Suppressive effects of mustard tissues are ascribed to the production of glucosinolates, which, upon mechanical disruption of the plant material, can be enzymatically degraded to form volatile antimicrobial compounds (Fenwick and Heaney, 1983).

Glucosinolates are organic anions consisting of a β-D-thioglucose moiety, a sulfonated oxime, and an aliphatic, heterocyclic, or aromatic carbon side-chain (Fenwick and Heaney, 1983; VanEtten and Tookey, 1983). Over 100 different glucosinolates have been identified in plants (Koritsas et al., 1991); they are found exclusively in dicotyledonous angiosperms (Kjaer, 1973; Fenwick and Heaney, 1983), mainly in the order Capparales, which includes the economically important family Brassicaceae (Ettlinger and Kjaer, 1968; Kjaer, 1973; VanEtten and Tookey, 1983). The magnitude of pathogen suppression by *Brassica* spp. is linked to the composition and concentration of glucosinolates, which can vary greatly among plant species, developmental stage, and tissue type (Kjaer, 1973; Fenwick and Heaney, 1983; Sang et al., 1984). Plant tissues of *B. juncea* (Indian mustard) were more suppressive to cereal pathogens when plants were harvested at flowering than at seed maturity (Kirkegaard et al., 1996). The formation of glucosinolates also depends on cultivation conditions, nutrient availability in the soil, and climatic conditions (Fenwick and Heaney, 1983; Glover et al., 1988; Duncan, 1991; Kirkegaard et al., 1996). Glucosinolates are hydrolyzed by the enzyme β-thioglucoside glucohydrolase, (EC 3.2.3.1; myrosinase) upon disruption of plant tissue. Besides production of D-glucose and $(SO_4)^{2-}$, hydrolysis results in formation of various volatile compounds with antimicrobial and phytotoxic properties, including isothiocyanates, thiocyanates, nitriles, hydroxynitriles, and epithionitriles (Fenwick and Heaney, 1983; Delaquis and Sholberg, 1997). The formation of the final hydrolysis product is determined by the carbon side chain of the glucosinolate and by reaction conditions, such as pH, metallic ion concentration, and water availability (Duncan, 1991).

Isothiocyanates (ITCs) can be produced in large quantities by *Brassica* spp.; over 50 organic ITCs are described as glucosinolate hydrolysis products (Kjaer, 1973). Allyl glucosinolate (sinigrin), which is generally converted to the volatile

allyl isothiocyanate (AITC), is the predominant glucosinolate produced in cultivars of *B. nigra* and *B. juncea* (Daxenbichler et al., 1991); it is one of the most potent antimicrobial compounds formed by *Brassica* spp. (MacLeod, 1976; Fenwick and Heaney, 1983; Mari et al., 1993; Kirkegaard et al., 1996; Vaughn and Boydston, 1997). Only AITC completely inhibited mycelial growth and germination of five postharvest fruit pathogens in a study including a number of sulfur compounds (Mari et al., 1993). AITC-releasing shoot tissue of *B. juncea* 99Y-1-1 suppressed growth of five cereal pathogens in vitro and was more suppressive than shoot tissue from *B. napus* Oscar, which does not release AITC (Kirkegaard et al., 1996). Although glucosinolate content and release of glucosinolate degradation products have been assessed for most members of the Brassicaceae, only a few studies have determined how much variability exists within individual species of crucifers.

Helminthosporium solani Dur. & Mont. and *Verticillium dahliae* are two potato pathogens of great economic importance. *Helminthosporium solani*, which causes silver scurf on potato tubers, spreads rapidly in storage and may survive in soil (Jellis and Taylor, 1974; Hooker, 1981; Merida and Loria, 1994). *Verticillium dahliae*, the cause of vascular wilt of potato, can also survive in the soil for up to several years. Whereas *V. dahliae* has a wide host range (Hooker, 1981), *H. solani* exclusively colonizes potato tubers (Jellis and Taylor, 1974). Additionally, *H. solani* is very slow growing in culture, making in vitro research difficult. To reduce soil-borne inoculum of *Verticillium* spp. and other pathogens, soil fumigants are frequently applied to the field prior to planting. Soil fumigants will be less available in the future due to environmental hazards associated with their production and application (Ristaino and Thomas, 1997). Selecting *Brassica* spp. that produce antifungal volatiles could be an important step in developing green manure crops as soil fumigant alternatives. Assuming that mustard genotypes with high AITC release can suppress soil-borne plant pathogens, data on AITC concentration would allow efficient selection of genotypes for breeding programs. In a previous study, we evaluated AITC production and suppression of *Fusarium sambucinum* Fuckel by six different *Brassica* species. We reported that *B. nigra* and *B. juncea* produced the greatest amounts of AITC, and those mustards with greatest AITC release suppressed pathogen growth best. However, we evaluated only a few, randomly selected cultivars of each mustard species. We also demonstrated that *B. juncea* Cutlass, which produces large amounts of AITC, was fungicidal to five fungal pathogens in vitro (Mayton et al., 1996). In the present study, we screened a large number of diverse genotypes of *B. juncea* and *B. nigra* for the amount of AITC released from greenhouse-grown plants to determine accessions with maximum AITC production. In parallel, we assessed whether plant tissue from the same mustard accessions could suppress radial growth of the two model pathogens *V. dahliae* and *H. solani* in vitro, and whether AITC release correlated with suppression of these two fungi. We also

evaluated the glucosinolate profile of a subset of these genotypes to determine whether additional volatile ITCs were released.

METHODS AND MATERIALS

Plant Material and Fungal Cultures. Seeds from *B. nigra* (28 genotypes), *B. juncea* (35 genotypes), and *B. napus* (Midas) that originated from different locations worldwide were obtained from the Agriculture Canada Research Station, Saskatoon, Saskatchewan (Midas, Cutlass, Forge, Lethbridge) or from Institut für Pflanzengenetik und Kulturpflanzenforschung Gatersleben (IPK), Germany (Tables 1 and 2). Most accessions of *Brassica* spp. obtained from Germany were originally collected as wild species in Asia and Europe. *Brassica napus* Midas was included as non-AITC-producing control. Plants were grown in the greenhouse as described by Mayton et al. (1996). Fungal cultures of *H. solani* and *V. dahliae* originated from our own culture collection; they were stored (as sporulating mycelium) on glass-fiber filter paper (Whatman, Maidstone, UK). For long-term storage, fungal cultures were placed in the center of Petri dishes and allowed to overgrow pieces of sterilized glass-fiber filters, which had been placed on V8-agar (200 ml V8 juice, 5 g $CaCO_3$, and 14 agar in 1 liter distilled water). Glass-fiber pieces were then aseptically removed, transferred into sterilized coin envelopes, and air-dried in a laminar flow hood for 24 hr before storage at −20°C in a desiccator. *Verticillium dahliae* was cultured at room temperature for seven days, and *H. solani* was grown at 23°C for 20–24 days prior to use; both pathogens were cultured on clarified V8 juice agar. Cultures of *H. solani* were sporulating when used in bioassays.

Bioassays. Effects of volatiles released from *Brassica* spp. on radial growth of fungi were assessed in bioassays similar to those previously described (Mayton et al., 1996). All *Brassica* genotypes included in this study were grown at the same time and under identical growing conditions (light, temperature, and soil) in the greenhouse until flowering. Twenty five grams of leaf and stem tissue from flowering plants were rapidly macerated in a food processor and placed in a glass jar (500 ml), which was covered immediately with the bottom of a Petri plate (85 mm) containing V8 juice agar amended with streptomycin sulfate (100 mg/liter) and penicillin G (100,000 units/liter), and an agar plug (5 mm) that was transferred from a culture of *H. solani* or *V. dahliae*. Cultures of *H. solani* were sporulating when tested. The inverted bottoms of the Petri plates were sealed to the glass jar with two layers of Time Tape (TimeMed Labeling Systems Inc., Burr Ridge, Illinois) and incubated in the dark at 18°C. Controls included jars with plant tissue from rapeseed Midas and empty jars (unamended control). Radial growth of fungal cultures was measured at three-day intervals for three weeks (*H. solani*) or about six days, until the unamended control cultures had grown

TABLE 1. IN VITRO GROWTH SUPPRESSION OF *Verticillium dahliae* AND *Helminthosporium solani* AND AITC RELEASE FROM MACERATED LEAF TISSUE OF 35 GENOTYPES OF *Brassica juncea*[a]

Accession/ cultivar[b]	Country of origin[c]	Cultivar (variety)	Percent inhibition of *V. dahliae*[d]	Percent inhibition of *H. solani*[d]	AITC (mg/g dry plant tissue)[e]
57	Germany		26.9 ± 12.1	55.1 ± 6.6	0.00 ± 0.00
58	Germany		91.3 ± 4.2	42.0 ± 5.0	0.86 ± 0.14
59	(Bulgaria)		100.0 ± 0.0	100.0 ± 0.0	1.19 ± 0.02
60	China		89.3 ± 6.8	93.2 ± 4.3	1.14 ± 0.29
61	China		100.0 ± 0.0	100.0 ± 0.0	1.36 ± 0.28
63	Afghanistan		100.0 ± 0.0	100.0 ± 0.0	2.62 ± 0.96
64	China		25.8 ± 5.0	51.4 ± 1.7	0.23 ± 0.00
65	Afghanistan		100.0 ± 0.0	100.0 ± 0.0	1.22 ± 0.15
239	China		100.0 ± 0.0	100.0 ± 0.0	1.49 ± 0.20
240	(China)		75.8 ± 11.1	86.8 ± 5.9	0.72 ± 0.05
418	China		100.0 ± 0.0	100.0 ± 0.0	2.14 ± 0.20
424	Bulgaria		44.4 ± 1.9	57.7 ± 3.3	0.01 ± 0.01
427	Bulgaria		100.0 ± 0.0	100.0 ± 0.0	0.86 ± 0.01
429	(Sweden)		100.0 ± 0.0	100.0 ± 0.0	0.76 ± 0.12
433	China		100.0 ± 0.0	100.0 ± 0.0	0.50 ± 0.06
434	(Yugoslavia)		100.0 ± 0.0	100.0 ± 0.0	1.05 ± 0.09
436	China		100.0 ± 0.0	100.0 ± 0.0	1.32 ± 0.08
477	(China)		87.6 ± 5.6	100.0 ± 0.0	1.02 ± 0.06
485	China		37.8 ± 5.4	45.0 ± 1.9	0.45 ± 0.03
488	China		100.0 ± 0.0	100.0 ± 0.0	1.29 ± 0.16
967	(Soviet Union)	Steart	100.0 ± 0.0	100.0 ± 0.0	1.39 ± 0.17
968	Soviet Union	Zaria	100.0 ± 0.0	100.0 ± 0.0	2.43 ± 0.72
973	Soviet Union	Druznaja	100.0 ± 0.0	100.0 ± 0.0	1.15 ± 0.08
976	China	Ta Tou Tsai	70.5 ± 13.2	100.0 ± 0.0	0.96 ± 0.15
1036	India	Mustard	31.4 ± 3.8	52.9 ± 7.1	0.07 ± 0.03
1160	China		36.8 ± 3.2	50.8 ± 2.7	0.00 ± 0.00
1201	Soviet Union	var. *crispifolia*	38.8 ± 3.7	100.0 ± 0.0	0.38 ± 0.17
1202	China		100.0 ± 0.0	100.0 ± 0.0	1.82 ± 0.17
1206	(Canada)	Stoke	65.5 ± 15.4	78.9 ± 9.5	1.88 ± 0.85
1207	(Canada)	Domo	94.2 ± 2.6	91.2 ± 5.6	0.95 ± 0.28
1285	Cuba		100.0 ± 0.0	100.0 ± 0.0	2.18 ± 0.45
1326	Great Britain	Green in the snow	100.0 ± 0.0	83.1 ± 7.5	0.97 ± 0.11
Cutlass	Canada	Cutlass	100.0 ± 0.0	100.0 ± 0.0	1.80 ± 0.23
Forge	Canada	Forge	100.0 ± 0.0	100.0 ± 0.0	1.39 ± 0.01
Lethbridge	Canada	Lethbridge	64.5 ± 1.0	65.7 ± 1.4	0.46 ± 0.03

[a]Disease suppression data present percent growth inhibition compared to unamended controls.
[b]Accession numbers for seeds obtained from Institut für Pflanzengenetik und Kulturpflanzenforschung (IPK), Gatersleben, Germany.
[c]Countries listed in parentheses name location where IPK obtained seeds, but country of origin is not known.
[d]Mean values from six replicates ± standard error, combined from two experiments.
[e]Mean values from two AITC assessments ± standard error.

TABLE 2. IN VITRO GROWTH SUPPRESSION OF *Verticillium dahliae* AND *Helminthosporium solani* AND AITC RELEASE FROM MACERATED LEAF TISSUE OF 28 GENOTYPES OF *Brassica nigra*[a]

Accession/ cultivar[b]	Country of origin[c]	Percent Inhibition of		AITC (mg/g dry plant tissue)[e]
		V. dahliae[d]	*H. solani*[d]	
21	Greece	70.4 ± 13.3	84.6 ± 6.9	0.95 ± 0.62
23	Greece	100.0 ± 0.0	100.0 ± 0.0	1.22 ± 0.10
24	Bulgaria	100.0 ± 0.0	100.0 ± 0.0	0.73 ± 0.13
25	Bulgaria	64.2 ± 16.1	98.0 ± 0.9	0.58 ± 0.03
26	Italy	100.0 ± 0.0	100.0 ± 0.0	1.02 ± 0.05
27	Italy	76.2 ± 10.7	80.2 ± 9.0	0.49 ± 0.29
30	Greece	100.0 ± 0.0	100.0 ± 0.0	1.20 ± 0.26
31	Greece	100.0 ± 0.0	100.0 ± 0.0	2.00 ± 0.20
33	Greece	41.9 ± 13.5	68.6 ± 4.0	0.39 ± 0.06
185	Greece	100.0 ± 0.0	100.0 ± 0.0	1.30 ± 0.07
186	Greece	100.0 ± 0.0	100.0 ± 0.0	1.56 ± 0.89
187	Greece	91.9 ± 3.75	79.0 ± 9.5	0.93 ± 0.44
188	Greece	100.0 ± 0.0	100.0 ± 0.0	1.43 ± 0.08
189	Greece	100.0 ± 0.0	100.0 ± 0.0	2.08 ± 1.53
190	Greece	46.7 ± 4.8	60.2 ± 1.1	0.51 ± 0.27
192	Greece	100.0 ± 0.77	100.0 ± 0.0	1.68 ± 0.89
331	unknown	89.0 ± 5.0	100.0 ± 0.0	0.86 ± 0.23
1044	Italy	100.0 ± 0.0	100.0 ± 0.0	1.16 ± 0.24
1045	Italy	100.0 ± 0.0	100.0 ± 0.0	0.96 ± 0.01
1046	Italy	100.0 ± 0.0	100.0 ± 0.0	2.06 ± 0.13
1163	Ethiopia	70.4 ± 2.6	58.4 ± 2.3	0.84 ± 0.03
1164	Italy	100.0 ± 0.0	100.0 ± 0.0	3.54 ± 0.65
1165	Ethiopia	100.0 ± 0.0	100.0 ± 0.0	1.52 ± 0.20
1270	(Germany)	100.0 ± 0.0	100.0 ± 0.0	1.59 ± 0.06
1434	(Germany)	100.0 ± 0.0	100.0 ± 0.0	2.51 ± 1.30
1437	(Poland)	32.74 ± 5.0	63.1 ± 7.2	0.60 ± 0.04
1438	(France)	100.0 ± 0.0	100.0 ± 0.0	2.94 ± 1.66
1440	(Italy)	100.0 ± 0.0	100.0 ± 0.0	1.51 ± 0.13

[a]Disease suppression data present percent growth inhibition compared to unamended controls.
[b]Accession number for seeds obtained from Institut für Pflanzengenetik und Kulturpflanzenforschung (IPK), Gatersleben, Germany.
[c]Countries listed in parentheses name location where IPK obtained seeds, but the country of origin is not known.
[d]Mean values from six replicates ± standard error, combined from 2 experiments.
[e]Mean values from two AITC assessments ± standard error.

to the edge of the plate (*V. dahliae*). Each mustard accession was tested for suppression of each fungus with three replicate plates per treatment; the entire experiment was performed twice. Growth inhibition (percent inhibition relative to the unamended control) was analyzed using ANOVA (data transformed to

square root prior to statistical analysis to fulfill assumptions of normality of data) with a linear model for nested and crossed factors (Neter et al., 1990; Kuehl, 1994). AITC production was compared to pathogen inhibition by regression analysis. Analyses were performed for individual experiments and repeated with combined data from both experiments, since means and variances did not differ significantly between experiments. All statistical analyses were performed using SAS software (SAS Institute, Cary, North Carolina). Whenever the growth of fungal cultures was completely suppressed by the mustard treatment, original mycelial plugs were transferred to new V8 juice agar plates and tested for viability. Radial growth measurements from rapeseed control treatments could not be included in statistical analyses, since fungal cultures in these treatments were frequently contaminated in bioassays with fungi originating from the plant material.

Glucosinolate Analysis by Gas Chromatography. Glucosinolates were extracted according to a modified protocol described by Mayton et al. (1996). Plant material was collected from the same plants grown for bioassays, rapidly frozen at −70°C, stored at −15°C for two weeks, and lyophilized prior to glucosinolate analysis. Glucosinolates were extracted from 5 g of ground, lyophilized leaf tissue representing a composite of leaves from three to six plants. Leaf tissue was blended with 30% aqueous methanol and shaken on a wrist shaker for 1 hr. The slurry was filtered through filter paper (Whatman No. 2), and the methanol removed by rotoevaporation. The aqueous residue was transferred to a centrifuge tube (50 ml) and mixed with 2 ml of a 1 : 1 solution of barium and lead acetate (0.5 M each) before centrifugation at 3200 *g* for 5 min. The supernatant was removed, frozen, and lyophilized, and the residue was resuspended in 15 ml of sodium phosphate buffer (0.05 M, pH 7.5). Dichloromethane (CH_2Cl_2, 15 ml) containing 1 mg/ml of 99% butyl isothiocyanate internal standard (Aldrich Chemical Co., Milwaukee, Wisconsin) was added, followed by addition of 25 mg thioglucosidase (Sigma Chemical Co., St. Louis, Missouri). The suspension was shaken at 21°C for 2 hr. The CH_2Cl_2 fraction containing glucosinolate degradation products was partitioned in a separatory funnel and analyzed on a Hewlett-Packard (HP) 5972A Series gas chromatograph equipped with a HP 5972A Mass Selective Detector (Hewlett-Packard Co., Palo Alto, California). Concentrations of glucosinolate degradation products were calculated based on peak areas in relation to the peak of a butyl isothiocyanate internal standard.

Glucosinolate Hydrolysis Product Analysis Using Solid Phase Microextraction. The relative proportions of volatile isothiocyanates released by 41 *Brassica* genotypes (Tables 3 and 4) were determined by headspace analysis. Brassica leaf and stem tissue (2.5 g) was harvested from flowering plants, macerated with mortar and pestle after addition of silica sand and distilled water (dH_2O) for a few seconds, and rapidly transferred to a 500-ml glass beaker with additional dH_2O. A Teflon stirring bar was added and two layers of aluminum foil were

TABLE 3. VOLATILES RELEASED FROM MACERATED GREEN TISSUE OF 21 *Brassica nigra* GENOTYPES AS DETERMINED BY HEADSPACE ANALYSIS (EXPRESSED AS PERCENT OF TOTAL ITC)

Genotype (accession)[a]	AITC (%)[b]	2-Phenylethyl-ITC (%)[b]	Other compounds
22	100		
25	100		
26	92	8	
27	96	4	
30	76	24	
185	94	6	
186	85	15	
187	81	10	*cis*-3-hexen-1-yl acetate
188	83	17	
189	78	22	
190	72	18	*cis*-3-hexen-1-yl acetate
331	91	9	
1044	81	19	
1046	82	13	*cis*-3-hexen-1-yl acetate
1163	67	33	
1164	96	4	
1165	11	89	
1270	55	38	*cis*-3-hexen-1-yl acetate
1434	63	37	
1437	73	27	
1440	100	0	

[a]Accession numbers for seeds obtained from Institut für Pflanzengenetik und Kulturpflanzenforschung (IPK), Gatersleben, Germany.
[b]Means of two replicates (expressed as % of overall ITCs). Only compounds comprising ≥4% of total volatiles are presented.

tightly wrapped over the opening of the beaker. A solid-phase microextraction (SPME) device (Supelco Inc., Bellefonte, Pennsylvania) with a 100-μm polydimethylsiloxane coating was inserted through the foil and allowed to adsorb volatiles for 15 min from the constantly stirring plant–water mixture. Adsorbed compounds were desorbed at 200°C onto the inlet injector for 1 min on a HP 5972A gas chromatograph connected to a HP 5972A mass selective detector. The analysis was conducted using a HP-5MS capillary column (30.0 m × 0.25 mm ID, 0.25-μm film thickness). Standards for isothiocyanates were run in a similar manner; each compound was added to a 500-ml flask with 5 ml of dH_2O to simulate extraction conditions for leaf tissue. The experiment was repeated, and data were pooled for statistical analyses. Compounds were identified by comparison of mass spectra with the standard, with previously published data for glucosinolate degradation products (Spencer and Daxenbichler, 1980), and with a library

TABLE 4. VOLATILES RELEASED FROM MACERATED GREEN TISSUE OF 21 *Brassica juncea* GENOTYPES AS DETERMINED BY HEADSPACE ANALYSIS (EXPRESSED AS PERCENT OF TOTAL ITC)

Genotype (accession)[a]	Allyl-ITC (%)[b]	2-Phenylethyl-ITC (%)[b]	3-Butenyl-ITC (%)[b]	Benzyl-ITC (%)[b]	Other compounds[c]
57	43	57			
58	52	19		29	
239	90	10			
240	92	8			
418	80	8			(*E*)-2-Hexenal
427	88	12			
433	89	11			
477	35	60			(*E*)-2-Hexenal
485	100				
488	72	28			
973	100				
1036				100	
1160			100		
1202	91	9			
1206	91	9			
1285	95	5			
1326	100				
Cutlass	100				
Forge	92	8			
Lethbidge	89	11			

[a]Accession numbers for seeds obtained from Institut für Pflanzengenetik und Kulturpflanzenforschung (IPK), Gatersleben, Germany.
[b]Means of two replicates (expressed as % of overall ITCs). Only compounds comprising ≥4% of total volatiles are presented.
[c]Compounds comprised less than 10% of overall ITCs.

database. Only volatiles representing ≥1% of overall glucosinolates produced were considered significant and are listed.

RESULTS

Bioassays. Volatiles from all accessions of *B. juncea* and *B. nigra* significantly ($P < 0.001$) reduced radial growth of both *V. dahliae* and *H. solani* compared to the control (Tables 1 and 2). Radial growth suppression differed significantly ($P < 0.001$) among *Brassica* genotypes within each *Brassica* species. Plant tissue of 57% of all *B. juncea* genotypes and 68% of all *B. nigra* genotypes was fungicidal to *V. dahliae* and *H. solani* in repeated experiments.

AITC Production. Quantities of AITC released by Brassica genotypes differed significantly ($P < 0.001$) and ranged from 0 to 3.54 mg AITC/g lyophilized plant tissue. All mustard accessions that fully inhibited fungal growth were also

fungicidal in bioassays, and released >0.7 mg AITC/g dry tissue (except for *B. juncea* 433). AITC was produced by all genotypes of *B. nigra* tested. Four genotypes of *B. juncea* (accessions 57, 424, 1036, 1160) produced no or very small amounts of AITC, but were inhibitory to pathogen growth (27–58% growth inhibition compared to unamended controls). Geographic origin of mustard accessions was not correlated to pathogen suppression or AITC production (Tables 1 and 2).

Radial growth suppression of *V. dahliae* and *H. solani* by all mustard genotypes was correlated to AITC concentration (Figure 1). Above a threshold release

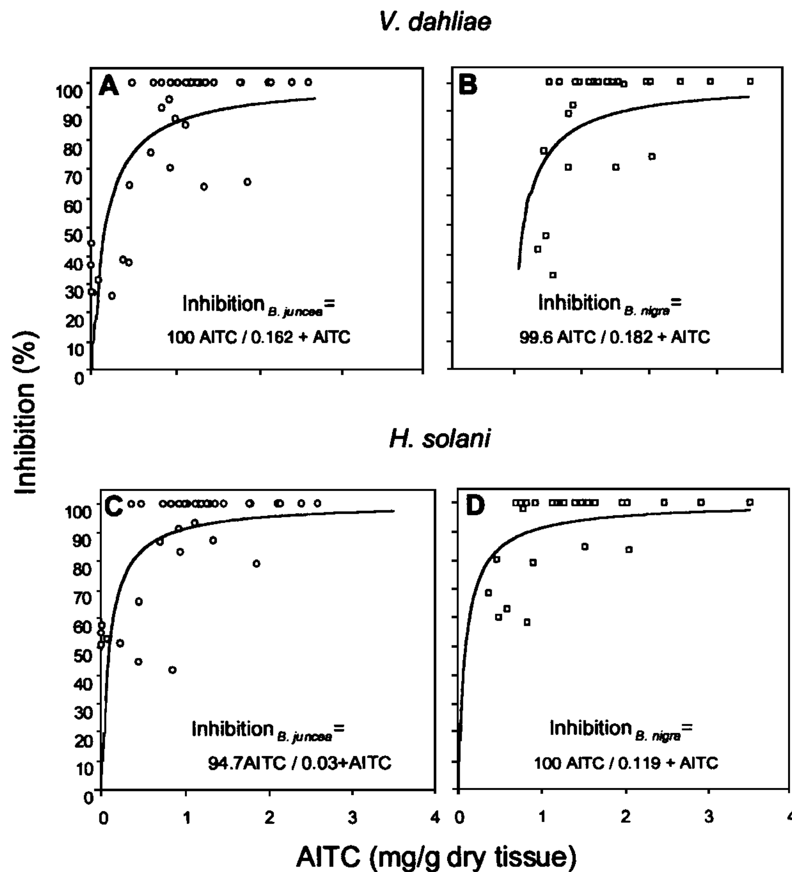

FIG. 1. Relationship between radial growth inhibition (compared to the unamended control) of *Verticillium dahliae* (A, B) or *Helminthosporium solani* (C, D) and AITC production by *B. juncea* (A, C) and *B. nigra* (B, D).

of 1.2 mg AITC/g dry plant tissue (which equals 3.5 mg of total AITC release per jar in bioassays), genotypes of either mustard species were consistently fungicidal to both pathogens (Tables 1 and 2). Rapeseed Midas did not produce AITC nor reduce radial growth of fungi; fungal growth was similar to that of the unamended controls (data not shown).

Glucosinolate Hydrolysis Product Analysis Using Solid-Phase Microextraction. Glucosinolate hydrolysis products released by a subset of *B. nigra* genotypes (21 genotypes) and *B. juncea* genotypes (20 genotypes) were determined by headspace analysis. Three different volatile ITCs were produced by *B. nigra* genotypes (Table 3) and four by *B. juncea* genotypes (Table 4). All accessions of *B. nigra* produced AITC after maceration of the green plant tissue, which comprised 11–100% of the total ITCs released. In addition, 86% of *B. nigra* genotypes tested released 2-phenylethyl-ITC; 19% produced *cis*-3-hexen-1-yl acetate (Table 3). Of the 20 *B. juncea* genotypes tested, 90% produced AITC, which comprised 35–100% of total ITCs released. 2-Phenylethyl-ITC was released by 70% of genotypes tested and comprised 5–100% of total ITCs. Benzyl-ITC was released by two genotypes (10%) and 3-butenyl-ITC by one genotype (5%) of *B. juncea* (Table 4).

DISCUSSION

This study evaluated AITC production in relation to pathogen suppression for 63 accessions of *B. juncea* (Indian mustard) and *B. nigra* (black mustard). We identified genotypes that release AITC in varying amounts; some genotypes released more AITC than previously reported for these species (Spak, 1988; Chin and Lindsay, 1993; Kirkegaard et al., 1996). Production of AITC varied greatly within each *Brassica* species. Overall, AITC release varied more across genotypes of *B. nigra* (0.4–3.5 mg AITC/g tissue) than *B.juncea* (0–2.6 mg AITC/g tissue). In a previous study, we compared five genotypes of *B. juncea* and four genotypes of *B. nigra* and reported similar differences in AITC release within each species (Mayton et al., 1996). Large variation in release of volatile sulfur compounds among cultivars has also been described for other *Brassica* spp. (Chin and Lindsay, 1993).

The data presented here demonstrate a correlation between AITC release and suppression of the two fungal pathogens, *H. solani* and *V. dahliae*, with different growth habits. The finding that mustards producing high levels of AITC were fungicidal was expected and is consistent with other reports describing fungicidal and nematicidal effects of AITC (Lazzeri et al., 1993; Mari et al., 1993; Mojtahedi et al., 1993). In our study, 17 of 28 *B. nigra* accessions released AITC in concentrations greater than 1.2 mg/g, and completely prevented fungal growth and spore germination.

Although both *V. dahliae* and *H. solani* were negatively affected by AITC-producing mustard accessions, genotypes releasing concentrations of AITC <1 mg/g tissue more efficiently reduced radial growth of the slow-growing *H. solani* than of the more rapidly growing *V. dahliae*. Whereas low AITC concentration coincided with poor pathogen suppression in most genotypes of *B. juncea*, a few genotypes produced little or no AITC but still reduced pathogen growth. This finding can be explained by the release of glucosinolate hydrolysis products other than AITC, e.g., benzyl-ITC, 3-butenyl-ITC, and 2-phenylethyl-ITC, that we identified in headspace analysis, which can also have allelopathic, fungicidal, or nematicidal effects (Duncan, 1991; Fenwick and Heaney, 1983; Vaughn and Boydston, 1997). Production of large amounts of benzyl-ITC by *B. juncea* accession 1036 and of 3-butenyl-ITC by accession 1160 could explain the growth suppression of *V. dahliae* and *H. solani* by these genotypes.

The major glucosinolate hydrolysis product released from *B. nigra* in this study was AITC, which confirms reports of others (Daxenbichler et al., 1991; Vaughn and Boydston, 1997). Most black mustard genotypes tested also released 2-phenylethyl-ITC in significant amounts, which likely contributed to pathogen suppression (Angus et al., 1994; Kirkegaard et al., 1996) and may be responsible for fungicidal activity in some of those genotypes that released AITC in amounts below 1.2 mg/g dry tissue. Release of 2-phenylethyl-ITC by *B. nigra* has been reported previously for a limited number of genotypes (Cole, 1976). Bialy et al. (1990) had previously shown that 2-phenylethyl-ITC was more potent than AITC in suppression of wheat seed germination. Others reported that *B. juncea* roots released 2-phenylethyl-ITC, which resulted in growth suppression of the take-all fungus, *Gaeumannomyces graminis* (Sacc.) Arx and Oliver var. *tritici* (Angus et al., 1994). We have demonstrated here that many *B. juncea* genotypes, as well as most genotypes of *B. nigra,* which have agronomic characteristics suitable for use as green manure crops, contain significant amounts of 2-phenylethyl-ITC in leaf tissue.

Although we did not evaluate roots of all mustard genotypes for production of sulfur compounds, it is likely that they release AITC and other fungitoxic volatiles (Angus et al., 1994; Kirkegaard et al., 1996), possibly resulting in an even greater potential for suppression of soil-borne pathogens than demonstrated in this study.

We observed small differences in AITC release when the same mustard genotype was grown at different times in the greenhouse (data not shown). These differences were likely caused by varying light conditions and small temperature changes in the greenhouses during plant growth (Kirkegaard et al., 1996). In field studies, we also observed small differences in AITC release by a mustard genotype planted at the same time at different field sites located in close proximity (data not shown).

Conflicting results exist on the suppressiveness of *B. napus*. A number

of reports describe that rapeseed can suppress pathogen growth (Mojtahedi et al., 1993; Williams-Woodward et al., 1997). In contrast, the high glucosinolate rapeseed cultivar Midas did not reduce radial growth of fungi compared to unamended controls and did not release AITC from leaf tissue in our study. This result confirms our previous findings (Mayton et al., 1996) and those of others describing superior pathogen or weed suppression with *B. juncea* cultivars compared to rapeseed or other *Brassica* spp. (Woods et al., 1991; Lazzeri et al., 1993; Kirkegaard et al., 1994, 1996; Vaughn and Boydston, 1997). Even though *B. napus* genotypes do not release AITC from green plant tissue, it is known that they can form other glucosinolate degradation products in varying amounts, which may have variable effects on pathogen growth.

This study describes the suppression of pathogen growth by *B. juncea* and *B. nigra* genotypes in vitro. We also tested the effect of several *B. juncea* genotypes against soil-borne diseases on legumes in the field. These genotypes had released great amounts of AITC and suppressed pathogen growth in vitro in our greenhouse studies. The genotypes also released great amounts of AITC when grown in the field, resulting in a significant reduction of disease severity on peas and snap beans (data not shown). Although the growing conditions may influence the release of AITC, no significant differences were detected among AITC release from greenhouse-grown and field-grown *Brassica* plants in our studies (data not shown).

Brassica sp. with high glucosinolate content have the potential to suppress germination of crop seeds (Bialy et al., 1990). However, Papavizas (1966) reported that emergence of peas was not affected by high AITC mustards when planted at least seven weeks after incorporation of the tissue, and Kirkegaard et al. (1994) demonstrated that wheat emergence was not reduced when planted only four weeks after *Brassica* tissue incorporation. We did not observe a negative effect on the emergence or growth of legumes planted four to five months after incorporation of *Brassica* tissue into the soil (data not shown).

Breeding efforts in recent decades have concentrated on the production of *B. juncea* and *B. napus* cultivars with low glucosinolate content for human consumption and animal feed; therefore, few high glucosinolate cultivars are available commercially. *Brassica nigra* was not included in recent breeding efforts. Our study included several commercially available cultivars of *B. juncea*, some of which produced high quantities of AITC, e.g., Stoke, Cutlass, and Forge. These cultivars may be useful as green manure crops with antifungal activity that do not require further improvement through breeding. In addition, we included genotypes collected in the wild in our study; these represent an important resource for breeding a variety of high-glucosinolate mustards with desirable agronomic characteristics for specific use as green manure crops. Mustard genotypes tested in this study originated in geographically distinct locations worldwide and have the advantage of being adapted to a wide range of climatic condi-

tions. The efficacy of *Brassica* green manure applications for disease suppression depends on the life-cycle stage of the pathogen present at time of application. Future studies should test the effect of AITC and other ITCs on long-term survival of the plant pathogens in soil. Of particular interest should be microsclerotia of *V. dahliae*, which are the major means of soil survival for this pathogen.

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SYNTHESIS AND DIGESTIBILITY INHIBITION OF DIARYLHEPTANOIDS: STRUCTURE–ACTIVITY RELATIONSHIP

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Abstract—(±)-5-Hydroxy-1,7-bis-(4′-hydroxyphenyl)-3-heptanone (**2a**), (±)-5-hydroxyl-1-(4′-hydroxyphenyl)-7-phenyl-3-heptanone (**2b**), (±)-5-hydroxy-7-(4′-hydroxyphenyl)-1-phenyl-3-heptanone (**2c**), and (±)-5-hydroxy-1,7-bis-(phenyl)-3-heptanone (**2d**) have been synthesized to study the structure–activity relationship regarding digestibility inhibition in vitro in cow rumen fluid. The activities were compared with the activity of chiral (*S*)-**2a** and its glucoside platyphylloside (**1**), isolated from *Betula pendula*. Compound **2a** was slightly less active, **2b** and **2c** were more active, and **2d** was less active than (*S*)-**2a** and platyphylloside.

Key Words–Platyphylloside, 5-hydroxy-3-platyphyllone, centrolobol, phenols, rumen fluid, birch bark, *Betula pendula*.

INTRODUCTION

Birch (*Betula pendula* Roth.) bark contains a phenolic glucoside, platyphylloside (**1**), that has been shown to reduce the in vitro digestibility of hay in goat and sheep rumen fluid up to 50% (Sunnerheim-Sjöberg et al., 1988).

Platyphylloside was first isolated by Terasawa et al. (1984) from *Betula platyphylla*. Diarylheptanoid glycosides have also been found in other species such as *Acer* spp. (Nagai et al., 1990; Nagumo et al., 1996), *Alnus* spp. (Sasaya and Izumiyama, 1974; Sasaya, 1985; Nomura et al., 1981; Ohta et al., 1984), *Curcuma xanthorrhiza* (Claeson et al., 1996), *Alpinia blephaocalyx* (Kadota et al., 1996), and *Zingiber officinale* (Kikuzaki and Nakatani, 1996). Many of

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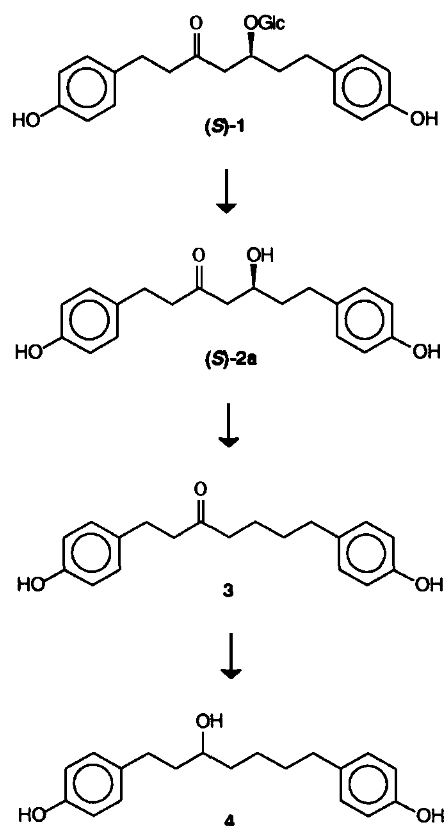


SCHEME 1. Metabolism of platyphylloside [(*S*)-**1**] in vitro in sheep rumen fluid. Platyphylloside [(*S*)-**1**] → (5*S*)-hydroxy-3-platyphyllone [(*S*)-**2a**] → 3-platyphyllone (**3**) → centrolobol (**4**).

them possess biological activities, such as antiinflammatory and antihepatotoxic effects, and some inhibit prostaglandin biosynthesis (Claeson et al., 1996, and references therein).

The metabolism of platyphylloside in vitro has been studied by Sunnerheim-Sjöberg and Knutsson (1995). When platyphylloside was added to hay and incubated together with rumen fluid, the metabolism was deduced to occur in three steps. First, hydrolysis of the glycosidic bond results in the hydroxy ketone 5-hydroxy-3-platyphyllone [(*S*)-**2a**]. Then, a reduction of the hydroxy function to a methylene group gives the ketone 3-platyphyllone (**3**). The carbonyl group is further reduced to a hydroxy functon leading to the alcohol centrolobol (**4**) (Scheme 1).

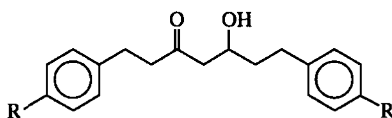


FIG. 1. Synthesized diarylheptanoids. **2a:** R = OH, R′ = OH, 5-hydroxy-1,7-bis-(4′-hydroxyphenyl)-3-heptanone; **2b:** R = OH, R′ = H, 5-hydroxy-1-(4′-hydroxyphenyl)-7-phenyl-3-heptanone; **2c:** R = H, R′ = OH, 5-hydroxy-7-(4′-hydroxyphenyl)-1-phenyl-3-heptanone; **2d:** R = H, R′ = H, 5-hydroxy-1,7-bisphenyl-3-heptanone.

The aim of this work was to synthesize four racemic diarylheptanoids (Figure 1) differing in the substituents on the aromatic rings, to study the structure–activity relationship regarding digestibility inhibition in vitro and to compare the activities to the activities of the chiral (*S*)-**2a** and its glucoside platyphylloside.

Linear diarylheptanoids containing the β-ketol function have been synthesized previously via direct C-alkylation by using diethyl phosphorocyanidate followed by Grignard reactions with aldehydes (Kato et al., 1984). In this paper, several analogues, **2a–d**, were synthesized via an aldol reaction. Our method provides a shorter, versatile route to this class of compounds.

METHODS AND MATERIALS

Chromatography

HPLC was performed on a Waters chromatograph equipped with a photo diode array detector. The column was a LichroCart 125-4 Lichrospher 100 RP-18 (5 μm) (Merck). The mobile phase consisted of solvent A: 0.01 M Ammonium formiate buffer, pH 3.4; and solvent B: acetonitrile. The gradient program was 0–10 min linear gradient from 50% to 70% B. The flow rate was 1 ml/min. TLC was performed on Merck HF-254 silica gel plates. For flash column chromatography, Merck Kieselgel 60 (230–400 mesh) was used.

Spectroscopy

^{1}H NMR (400 MHz) and ^{13}C NMR (100.5 MHz) NMR spectra were recorded on a Varian Unity 400 by using the solvent signals ($CDCl_3$ or CD_3OD) as internal standards.

Digestibility Experiment

In vitro organic matter digestibility (IVOMD) was determined according to standard methods originally described by Lindgren (1979). Rumen fluid was

sampled from a cannulated cow fed on 40% hay and 60% concentrate. The samples (see below) were incubated for 96 hr in 50 ml of a mixture of buffer (pH 6.8 ± 0.1) and rumen fluid (50 : 1) maintained under anaerobic conditions at 38°C in a water bath (Lindgren, 1979). After incubation, filtration, and washing, the amount of insoluble organic matter was determined by the difference in weight between dried (103°C over night) and deashed (500°C for 1.5 hr) solid residue. Reduction of digestibility was calculated by using equation 1:

$$(IVOMD_{ctrl} - IVOMD_{sample})/IVOMD_{ctrl} \quad (1)$$

Sample Preparation. To each tube, 500 mg milled hay and 0.050 mmol of the compound to be tested dissolved in 96% aqueous ethanol was added and mixed. (The concentration of the tested compounds in hay was, thus, about five times higher compared to the concentration of platyphylloside found in birch twigs and about the same as estimated in birch bark.) The control samples were treated the same way but with hay and solvent only. Three replicates of each sample were made. The solvent was allowed to evaporate at 40°C for 16 hr (all of the tested compounds were stable at this temperature). To each tube, 50 ml of buffer with 2% rumen fluid was added. After incubation (96 hr at 38°C), the hay residues were separated from the liquid by filtration. Ethanol (60 ml) was added to the different samples to stop enzymatic reactions during storage. Before HPLC analysis, the solvent was evaporated, and the residue was dissolved in water. The aqueous phase was extracted with ether/pentane (50 : 50) and washed with water and brine. The organic phase was dried with $MgSO_4$, and the solvent was evaporated.

Source of Chemicals

Platyphylloside [(5*S*)-hydroxy-1,7-bis-(4′-hydroxyphenyl)-3-heptanone-5-*O*-*β*-D-glucopyranoside (**1**)] was isolated from the inner bark of *Betula pendula* according to Smite et al. (1993). (5*S*)-Hydroxy-3-platyphyllone [(5*S*)-hydroxy-1,7-bis-(4′-hydroxyphenyl)-3-heptanone, (*S*)-**a**] was obtained by enzymatic hydrolysis of platyphylloside (Sunnerheim-Sjöberg et al., 1988).

4-(4′-Pyranyloxyphenyl)-2-butanone (***5a***). To a stirred solution of 4-(4′-hydroxyphenyl)-2-butanone (7.50 g, 46 mmol) and freshly distilled dihydropyran (10.5 ml, 113 mmol) in dry CH_2Cl_2 (40 ml) was added *p*-toluenesulfonic acid (monohydrate) (0.085 g, 0.44 mmol) in one portion. The mixture was stirred at room temperature for 1 hr. $NaHCO_3$ (aq) was added, and the mixture was stirred for 30 min. The mixture was extracted with Et_2O, dried over $MgSO_4$, and the solvent was evaporated to give a brown oil that was further purified by flash column chromatography (pentane–ether, 70 : 30) to give a colorless liquid (10.396 g, 92%). ^1H NMR was in accordance with literature (Schuster and Polowczyk, 1966).

Methyl 3-(4′-pyranyloxyphenyl)propanoate (***9***). To a stirred solution of

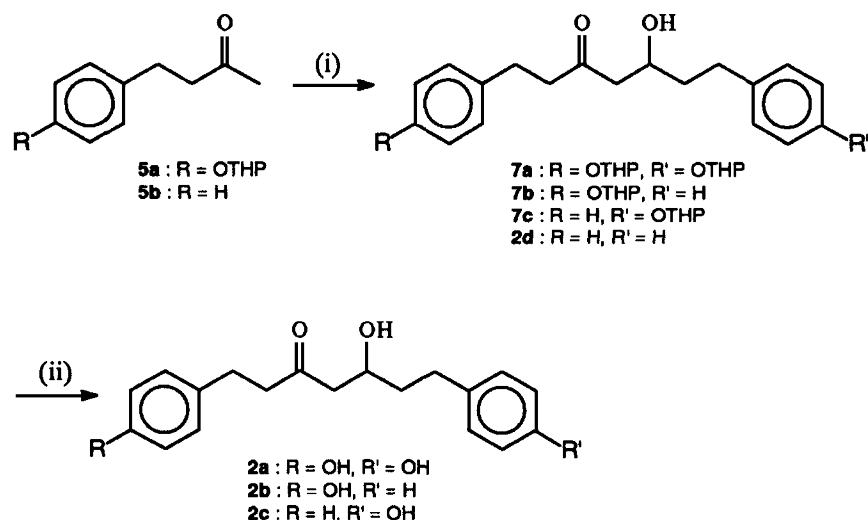
methyl 3-(4′-hydroxyphenyl)propanoate (**8**) (2.02 g, 11.2 mmol) was freshly distilled dihydropyran (2.6 ml, 28 mmol) in dry CH_2Cl_2 (10 ml) was added *p*-toluenesulfonic acid (monohydrate) (0.021 g, 0.11 mmol) in one portion. The mixture was stirred at room temperature for 1.5 hr. $NaHCO_3$ (aq) was added, and the mixture was stirred for 30 min. The mixture was extracted with Et_2O, dried over $MgSO_4$, and the solvent was evaporated to give a brown oil, which was further purified by flash column chromatography (pentane–ether, 60 : 40) to give a colorless liquid (3.198 g, 93%). ^{1}H NMR ($CDCl_3$) δ 7.10 (m, 2H), 6.98 (m, 2H), 5.37 (t, J = 3.2 Hz, 1H), 3.91 (m, 1H), 3.66 (s, 3H), 3.60 (m, 1H), 2.89 (m, 2H), 2.60 (m, 2H), 1.95–2.05 (m, 1H), 1.82–1.88 (m, 2H), 1.53–1.71 (m, 3H); ^{13}C NMR ($CDCl_3$) δ 173.4, 155.5, 133.5, 129.1, 116.5, 96.5, 62.0, 51.5, 36.0, 30.4, 25.2, 18.9.

*3-(4′-Pyranyloxyphenyl)propanol (**10**).* A solution of methyl-3-(4′-pyranyloxyphenyl)propanoate (**9**) (11.445 g, 43.4 mmol) in dry diethyl ether (20 ml) was added dropwise to a stirred suspension of $LiAlH_4$ (2.472 g, 65.0 mmol) in diethyl ether (100 ml) at room temperature. The mixture was left to stir under nitrogen atmosphere at room temperature for 1.5 hr. $Na_2SO_4 \times 10\ H_2O$ was added carefully in portions until no gas evolved. The mixture was filtered, the solid washed with ether, the filtrate dried with $MgSO_4$, and the solvent evaporated to give a colorless liquid (1.522 g, 97%). The product was used in the next step without further purification. ^{1}H NMR ($CDCl_3$) δ 7.10 (m, 2H), 6.98 (m, 2H), 5.37 (t, J = 3.4 Hz, 1H), 3.93 (m, 1H), 3.65 (t, J = 6.4 Hz, 2H), 3.60 (m, 1H), 2.64 (m, 2H), 1.95–2.07 (m, 1H), 1.82–1.92 (m, 2H), 1.57–1.73 (m, 3H); ^{13}C NMR ($CDCl_3$) δ 155.3, 134.8, 129.2, 116.5, 96.6, 62.2, 62.0, 34.3, 31.2, 30.4, 25.2, 18.8.

*3-(4′-Pyranyloxyphenyl)propanal (**6a**).* Pyridinium chlorochromate (0.660 g, 3.1 mmol) and sodium acetate (0.051 g, 0.6 mmol) were suspended in anhydrous CH_2Cl_2 (5 ml), and 3-(4′-pyranyloxyphenyl)propanol (**10**) (0.530 g, 2.2 mmol) in CH_2Cl_2 (5 ml) was added in one portion to the stirred solution. After 2 hr, dry ether was added, and the supernatant was decanted from the black insoluble residue. The residue was washed with ether, the combined organic phases were filtered through silica, and the solvent was evaporated to give a colorless liquid (0.343 g, 67%), which was pure according to ^{1}H NMR. ^{1}H NMR ($CDCl_3$) δ 9,81 (m, 1H), 7.07–7.13 (m, 2H), 6.98 (m, 2H), 5.38 (t, J = 3.2 Hz, 1H), 3.91 (m, 1H), 3.59 (m, 1H), 2.90 (m, 2H), 2.75 (m, 2H), 1.95–2.05 (m, 1H), 1.82–1.88 (m, 2H), 1.54–1.73 (m, 3H); ^{13}C NMR ($CDCl_3$) δ 201.7, 155.5, 133.3, 129.1, 116.6, 96.5, 62.0, 45.5, 30.4, 27.4, 25.2, 18.8.

General Procedure for Aldol Condensation: Synthesis of Compound 7a (Scheme 2)

To a flame-dried flask under nitrogen atmosphere, diisopropylamine (0.21 ml, 1.5 mmol) was added followed by dry THF (3.0 ml). After the mixture was cooled to −78°C, *n*-butyllithium (1.6 M, 0.89 ml, 1.4 mmol) was added, and



SCHEME 2. (i) 1) LDA (lithium diisopropylamide), 2) **6a** or **6b** (Figure 2), 3) H^+; (ii) HOAc–THF–H_2O (2 : 2 : 1).

the mixture was stirred for 35 min. A solution of 4-(4′-pyranyloxyphenyl)-2-butanone (0.321 g, 1.3 mmol) in THF (3.0 ml) was added slowly. After stirring for 30 min, a solution of 3-(4′-pyranyloxyphenyl)propanal (0.304 g, 1.3 mmol) in THF (2.0 ml) was added dropwise, and the mixture stirred for another 2 hr. The reaction was quenched at −78°C by careful addition of HCl (1 M, 5.0 ml). The reaction mixture was allowed to warm to room temperature. The mixture was extracted with ether, washed with brine, and dried with $MgSO_4$. The solvent was evaporated to give the crude product, which was purified by flash column chromatography (ether–pentane) to give a colorless oil.

Deprotection of THP

The deprotection of THP was done according to Bernady et al. (1979). Spectral data were consistent with those previously reported [**a** (Sunnerheim-Sjöberg and Knutsson, 1995), **2c** (Kiuchi et al., 1992), **2d** (Asakawa, 1970), **2b** ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.16–7.32 (m, 5H), 7.02 (m, 2H), 6.74 (m, 2H), 4.05 (oct, J = 4.0 Hz, 1H), 3.16 (d, J = 4.0 Hz, OH), 2.75–2.86 (m, 3H), 2.62–2.73 (m, 3H), 2.54 (m, 2H), 1.76–1.87 (m, 1H), 1.62–1.72 (m, 2H); ^{13}C NMR (100.5 MHz, $CDCl_3$) δ 211.7, 154.3, 141.5, 132.0, 129.2, 128.3, 125.8, 115.4, 67.2, 49.0, 45.2, 37.8, 31.6, 28.5. (Kiuchi et al., 1992)].

RESULTS AND DISCUSSION

Synthetic Route

The aldol condensation with the ketones (**5a,b**) and aldehydes (**6a,b**) produced the diarylheptanoides (**7a–c** and **2d**) containing the (±)-β-ketol skeleton as shown in Scheme 2. The phenolic moieties were protected as tetrahydropyranyl (THP) ethers. The yields from the aldol condensation reactions can be seen in Table 1.

The aldehyde **6a** (Figure 2) was prepared according to Scheme 3. Protection of the commercially available ester **8** with dihydropyrane (DHP) followed by reduction using lithium aluminum hydride ($LiAlH_4$) and oxidation using pyridinium chlorochromate (PCC) (Corey and Suggs, 1975) produced the aldehyde **6a**.

Digestibility Studies: Structure–Activity Relationship

Platyphylloside [(*S*)-1], (*S*)-**2a**, racemic **2a**, and the three analogs, **2b–2d** (Figure 1), were tested for digestibility inhibition in vitro in cow rumen fluid. The results are presented in Figure 3.

Glycoside–Aglycone. It is known from earlier studies (Sunnerheim-Sjöberg and Knutsson, 1995) that the glycosidic bond in platyphylloside is completely hydrolyzed in vitro within 6 hr in rumen fluid to (*S*)-**2a**. Moreover, the digestibility-reducing activity is correlated with the concentration of the metabolite (**4**) formed by reduction of (*S*)-**2a** (Sunnerheim-Sjöberg et al., 1988). Thus, the aglycone and its glucoside, platyphylloside, were expected to behave identi-

TABLE 1. ALDOL CONDENSATION OF KETONES **5a,b** AND ALDEHYDES **6a,b**

Entry	Ketone	Aldehyde	Product	Yield (%)
1	**5a**	**6a**	**7a**	71
2	**5a**	**6b**	**7b**	67
3	**5b**	**6a**	**7c**	72
4	**5b**	**6b**	**2d**	63

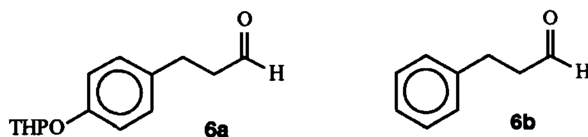
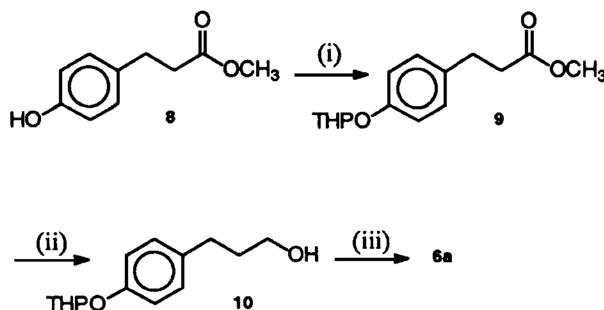


FIG. 2. **6a** = 3-(4′-Pyranyloxyphenyl)propanal, **6b** = 3-phenylpropanal.



SCHEME 3. (i) DHP, *p*-toluenesulfonic acid, CH_2Cl_2, 93%; (ii) $LiAlH_4$, Et_2O, 97% (iii) PCC, $NaCO_2CH_3$, CH_2Cl_2, 67%.

cally after a short incubation time. The results confirmed these expectations since the platyphylloside was almost as active as (*S*)-**2a** (32% and 35%, respectively, Figure 3). Furthermore, HPLC analysis of the rumen fluid after incubation showed, as expected from previous results (Sunnerheim-Sjöberg and Knutsson, 1995), that only two metabolites, **3** and **4**, were formed from both platyphylloside and (*S*)-**2a**. The concentration ratios of **4** and **3**, were 36 : 64 and 38 : 62 from **1** and (*S*)-**2a**, respectively.

Stereochemistry–Activity. The racemic mixture of **2a** was slightly less active than the isolated, optically active one, (*S*)-**2a**, 28% and 35%, respectively (Figure 3). The chromatograms (Figure 4a and 4b) show that (*S*)-**2a** is totally metabolized, while the racemic **2a** is not.

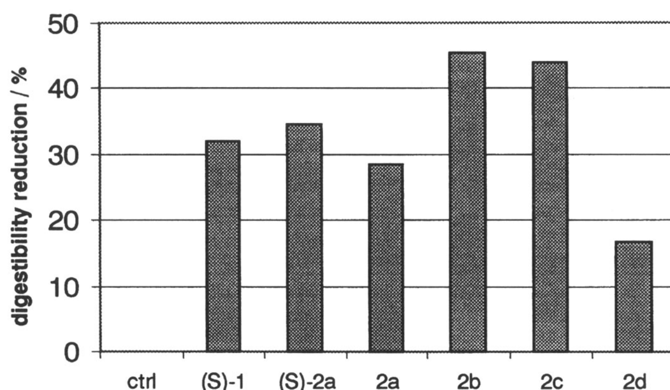


FIG. 3. Digestibility reduction after incubation for 96-hr. ctrl = control, (*S*)-**1** = platyphylloside, (*S*)-**2a** = (5*S*)-hydroxy-1,7-bis-(4′-hydroxyphenyl)-3-heptanone, **2a–d**, see Figure 1. Standard deviations: 0.75, 3.26, 1.07, 1.03, 0.46, 0.90, and 0.95, respectively.

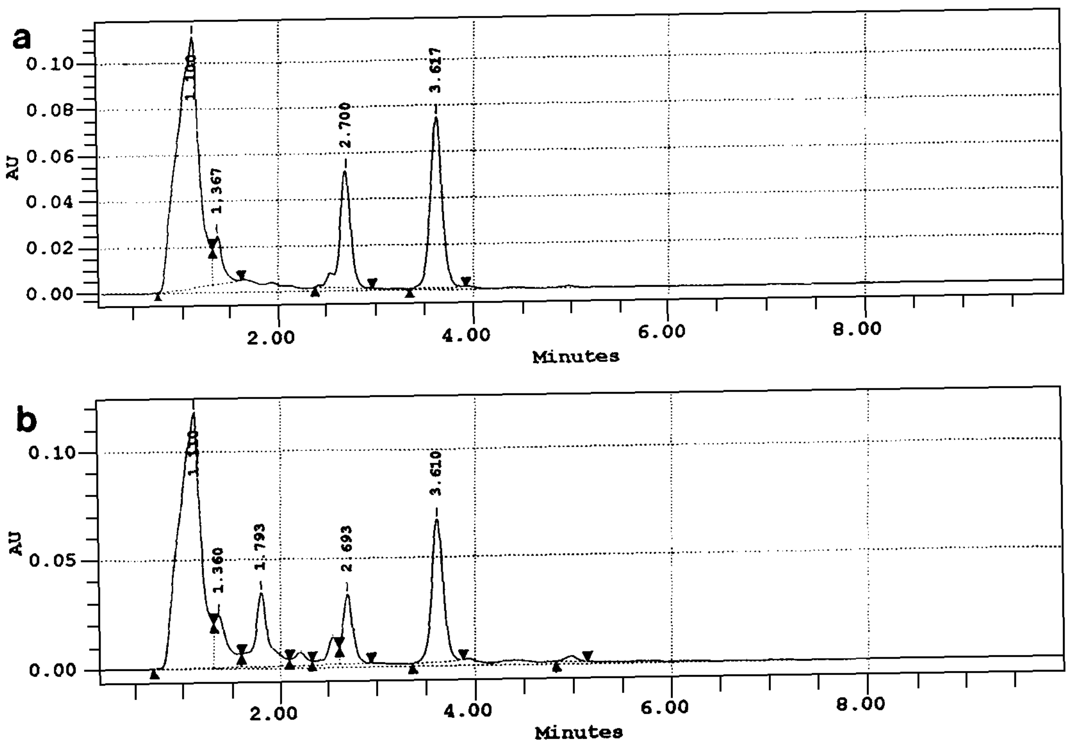
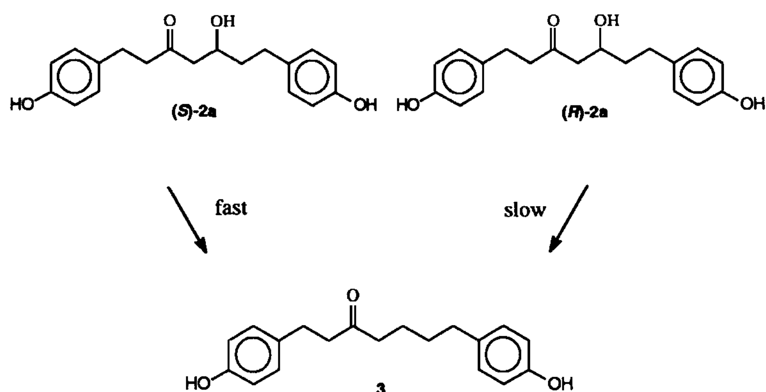


FIG. 4. (a) HPLC of rumen fluid after incubation with (5*S*)-hydroxy-3-platyphyllone [(*S*)-**2a**], R_t = 2.7 min centrolobol (**4**), R_t = 3.6 min 3-platphyllone (**3**). (b) HPLC of rumen fluid after incubation with (±)-5-hydroxy-3-platyphyllone (**2a**), R_t = 1.8 min 5-hydroxy-3-platphyllone, R_t = 2.7 min centrolobol (**4**) R_t = 3.6 min 3-platyphyllone (**3**).



SCHEME 4. In vitro metabolism of (*S*)-**2a** and (*R*)-**2a** in rumen fluid.

Estimation from peak areas shows that only about three fourths of the racemic compound has been metabolized. We assume that this is due to slower reduction of (*R*)-**2a** than to the *S*-enantiomer in the rumen fluid (Scheme 4).

Active Metabolite. The estimated relative areas of the peaks from centrolobol (**4**) in the chromatograms in Figure 4a and 4b are correlated to the activity. This supports the results from a previous study (Sunnerheim-Sjöberg and Knutsson, 1995) indicating centolobol (**4**) as the active metabolite.

Aromatic Substitution–Activity. The two derivatives lacking one phenolic hydroxyl group (**2b** and **2c**) possess a higher activity than the naturally occurring platyphylloside and its aglycone, while the nonphenolic derivative (**2d**) is less active (Figure 3).

SUMMARY

IVOMD of two isolated and four synthetic diarylheptanoids was studied for structure–activity relationship. Both the hydroxylation pattern in the aromatic rings and the stereochemistry were of importance for the activity.

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SEX PHEROMONE OF *Callosobruchus subinnotatus*

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Abstract—Sex pheromone produced by *Callosobruchus subinnotatus* females stimulates conspecific males to walk upwind toward the source. Gas chromatographic and mass spectrometric analyses of sex pheromone components and their hydrogenated derivatives suggested that the sex pheromone consisted of two short-chain fatty acids, (*E*)-3-methyl-2-heptenoic acid (E32C7) and (*Z*)-3-methyl-2-heptenoic acid (Z32C7). The composition of the sex pheromone was confirmed by electrophysiological and behavioral bioassays with synthetic compounds. A mixture of 5 ng E32C7 and 5 ng Z32C7 elicited a 2.1-mV male EAG response, whereas the solvent control elicited 0.1 mV. Mixtures of the two compounds at various ratios were attractive to males in Y-tube bioassays. A pitfall-type trap equipped with a lure impregnated with 1 ng E32C7 and 1 ng Z32C7 was effective in trapping males. Traps baited with the lure caught 80% of males in a glass aquarium during nighttime and 50% during daytime, whereas control traps (solvent treated) in a separate aquarium caught 20% and 10%, respectively.

Key Words—Short-chain fatty acid, 3-methyl-2-heptenoic acid, electroantennogram, stored-product pests, bruchids.

INTRODUCTION

Bambarra groundnut (*Vigna subterranea* L.) serves as a major source of dietary proteins for people in parts of western Africa (Rachie, 1985). The stored product insect, *Callosobruchus subinnotatus* (Pic), is a major pest inflicting heavy losses

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to stored bambarra groundnut (Mbata, 1991). A safe and inexpensive control measure has yet to be developed to control this pest. To improve the aesthetics of bambarra groundnuts for marketing, merchants in market places in Nigeria were observed to mix them with pesticides to kill *C. subinnotatus* (S. B. Ramaswamy, personal observation). Sex pheromone could be used as part of a safe and efficient pest management system for *C. subinnotatus*. Sex pheromones have been identified in two other *Callosobruchus* species (Cork et al., 1991; Phillips et al., 1996), and production of sex pheromone by female *C. subinnotatus* has been demonstrated (Shu et al., 1998). In this paper, we report the isolation and identification of *C. subinnotatus* sex pheromone.

METHODS AND MATERIALS

Insects. A culture of *C. subinnotatus* was maintained under quarantine at Mississippi State University on bambarra beans (groundnuts) in 1-liter widemouth glass jars at ca. 30°C and ca. 70% relative humidity and a light cycle of 12L : 12D. Infested beans were held individually in 11-ml glass shell vials for adult emergence. Vials were checked for newly emerged beetles three times a day to obtain virgin beetles. Male and female beetles were placed individually in shell vials with a bambarra bean. Voucher specimens (Lot Number 089) have been deposited in the Kansas State University Museum of Entomological & Prairie Arthropod Research.

Solvent Extraction of Females. Two-day-old virgin females were subjected to whole-body extraction by rinsing for a few seconds in hexane followed by diethyl ether. The hexane and ether extracts were pooled, concentrated under N_2, and resuspended in an appropriate amount of hexane to obtain concentrations in female equivalents (FE) per 10 μl hexane.

Isolation on Florisil in Open Column Chromatography. Female extracts were concentrated under N_2 and eluted through a Florisil column (deactivated with 7% water) sequentially with hexane, 10% ether in hexane (E/H), 25% E/H, 50% E/H, ether, and 4% formic acid in ether (4% FAE). Each fraction was dried under N_2 to remove solvents and resuspended in hexane.

Isolation by C-18-RP Open Column Chromatography. Female extracts were concentrated carefully under N_2, resuspended in 85% methanol in water (M/W), and loaded onto a C-18-RP (C-18 reverse phase) (35–75 μm, Alltech, Dearfield, Illinois) open column prewashed with methanol and 85% M/W. The column was eluted with 85% M/W followed by diethyl ether. The ether fraction was concentrated and resuspended in hexane. The 85% M/W fraction was dried carefully under N_2 to remove methanol and then extracted with diethyl ether. Ether extracts were dehydrated over $NaSO_4$, concentrated, and resuspended in hexane.

Isolation by Gas Chromatography (GC). Gas chromatography was performed with a 5700A Hewlett Packard (HP) GC equipped with a 30-m × 0.53-mm Econo-Cap Carbowax column (Alltech), a flame ionization detector (FID), an effluent splitter, and a thermal gradient collector [similar to that described by Brownlee and Silverstein (1968)]. Helium was used as the carrier gas at a linear rate of 40 cm/sec. Oven temperature was held at 60°C for 2 min, increased at the rate of 8°C/min to the final temperature of 220°C, and held for 10 min. The auxiliary oven temperature was set at 300°C.

Mass Spectrometry. Mass spectra were obtained on an HP 5890 series II Plus GC equipped with a 30-m × 0.25-mm Econo-Cap Carbowax column (Alltech) and coupled to an HP 5972 mass selective detector running in the EI (electron ionization) scan mode. Helium was used as the carrier gas at a constant flow rate of 1.0 ml/min (38.5 cm/sec linear rate). Oven temperature was held at 60°C for 2 min, increased at the rate of 10°C/min to the final temperature of 220°C, and held for 25 min.

Hydrogenation. The insect extracts were hydrogenated by passing hydrogen through the hexane solution over palladium (5% Pd on activated carbon). Following hydrogenation, the hexane was evaporated and hydrogenated products were redissolved with diethyl ether. Chemicals were deuterated in a similar manner.

Synthetics. Synthetic (*E*)- and (*Z*)-3-methyl-2-heptenoic acids and (*Z*)-3-methyl-3-heptenoic acid were synthesized as described by Cork et al. (1991).

Electroantennogram (EAG) and Behavioral Assays. EAG recordings were made as described by Ramaswamy et al. (1995). Males used for EAG recordings were 3 or 4 days old. Behavioral assays were conducted in 10-mm-ID Y-tubes with 8-cm-long arms and a 12-cm-long stem. The solvent extracts or synthetics in solvent were dispensed in 10-μl volumes to filter paper strips (4 mm × 1 mm), air-dried, and then placed in the upwind end of one arm. A paper strip treated with solvent alone and air-dried was placed in the upwind end of the other arm. The airflow rate through the Y-tube was measured at ca. 400 cc/min. Volumes of solvent solutions applied to filter papers were all 10 μl.

Pitfall Trap Assay. A trap device (20 cm long and 1.8 cm ID) was made from white PVC pipes. The device was similar in design to the "grain guard" pitfall trap (Burkholder and Ma, 1985), but with only three holes near the top. At the beginning of each period, day or night, 10 fresh male beetles were placed into a glass aquarium (40 cm long × 21 cm deep × 26 cm high) containing a trap with a rubber septum (sleeve type, 18 mm, Cat. No. 8753-D22, Thomas Scientific) newly impregnated with test chemicals in solvent or solvent alone. Chemicals for treatments were 1 ng each of synthetic (*E*)-3-methyl-2-heptenoic acid and (*Z*)-3-methyl-2-heptenoic acid. At the end of each observation period, the number of beetles inside the trap was recorded.

Statistical Analysis. Male EAG data were subjected to ANOVA and mul-

tiple comparisons (SAS for Windows, SAS Institute Inc.). Y-tube behavior data were subjected to χ^2 analysis. Expected values for calculating χ^2 were derived from results of male behavior responses in Y-tube olfactometers to hexane. The behavior test had three possible outcomes: choosing to enter either of the two arms or failing to enter either arm.

RESULTS AND DISCUSSION

Male EAG response to extracts containing 0.03 FE was 1.7 ± 0.15 mV, significantly higher than that to solvent control (0.1 ± 0.02 mV). A previous study showed that female *C. subinnotatus* produce and emit a sex pheromone. Male EAG responses to female extracts were log-linear (Shu et al., 1998) and could be used to monitor the activity of extracts.

After fractionation by Florisil open column chromatography, the 4% formic acid in ether (FAE) fraction elicited a significantly higher male EAG response than did any other fraction (Figure 1), indicating that 4% FAE could recover most, if not all, of the pheromone from the Florisil column. Standards tested with the same solvent system suggested that 4% FAE could elute heptanoic acids from a Florisil column, which could not be accomplished with ether alone. This

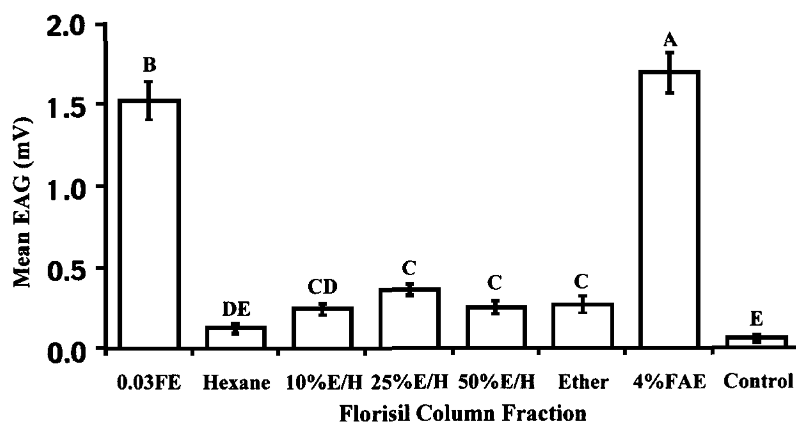

FIG. 1. Male EAG response (mean ± SE) to female extracts (0.5 FE) subjected to Florisil open column chromatography; 0.03FE is 0.03 female equivalent of crude extract; 10% E/H, 25% E/H, 50% E/H, and 4% FAE are 10% ether in hexane, 25% ether in hexane, 50% ether in hexane, and 4% formic acid in ether fractions of the Florisil column, respectively. Control is solvent. Different letters above bars indicate significance (LSD) at $\alpha = 0.05$ ($N = 6$).

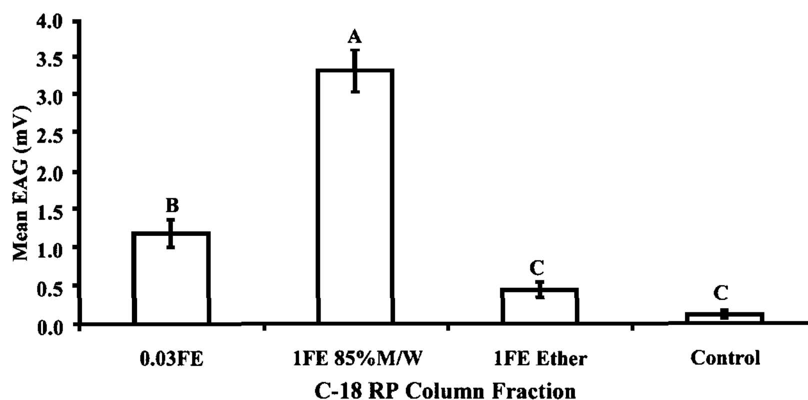

FIG. 2. Male EAG response (mean ± SE) to female extracts subjected to C-18-RP open column chromatography; 0.03 FE is 0.03 female equivalent of crude extracts. 1 FE 85% M/W and 1 FE Ether are 1 FE 85% methanol in water fraction and 1 FE ether fraction of C-18-RP column, respectively. Control is solvent. Different letters above bars indicate significance (LSD) at $\alpha = 0.05$ ($N = 8$).

latter finding implied that the components of sex pheromone of *C. subinnotatus* are aliphatic carboxylic acid(s). Other fractions elicited male EAG responses that were much lower than responses to the 4% FAE fraction but significantly higher than responses to the solvent control, and they were not pursued further.

Preliminary GC analysis indicated that the 4% FAE fraction contained large quantities of high-molecular-weight compounds, so large that in routine GC analysis, the GC column often was overloaded. Therefore, C-18-RP open column chromatography was used to fractionate female extracts. Male EAG responses to the latter fractions indicated that all pheromonal activity was recovered with 85% methanol in water (M/W). One FE of the 85% M/W fraction elicited a significantly higher male EAG response than did 1 FE of the ether fraction (Figure 2), which gave responses similar to solvent alone. Studies with standards indicated that 85% M/W recovered 100% of decanoic acid from the C18-RP column, while hexadecanoic acid remained on the column. This finding suggested that the sex pheromone components in *C. subinnotatus* are short-chain aliphatic acid(s).

The 85% M/W C-18-RP fraction was subjected to GC fractionation as indicated in Figure 3b. A total of five GC fractions was collected. Male EAG responses to fractions 2 and 3 at 2 FE were similar in magnitude and significantly higher than those to other GC fractions at the same dose and to the control (Figure 3a). This suggested that fractions 2 and 3 contained the putative sex pheromone components.

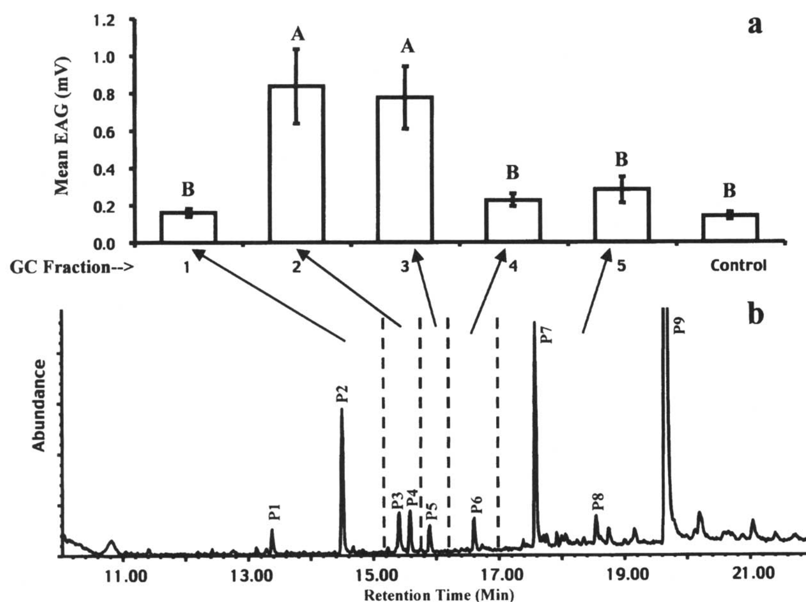

FIG. 3. Male EAG response (mean ± SE) to GC fractions (2 FE) of 85% M/W fraction of C-18-RP column and to solvent (Control) (a) and GC chromatogram (b). Different letters above bars in top figure (a) indicate significance (LSD) at $\alpha = 0.05$ ($N = 6$).

Major GC peaks were labeled as indicated in Figure 3b. Mass spectra were obtained for all major peaks from the 85% M/W C-18-RP fraction. Peak P3 had the same mass spectrum as synthetic (Z)-3-methyl-2-heptenoic acid (Z32C7). The synthetic Z32C7 coeluted with P3, confirming the identification of P3 as being Z32C7.

Peak P5 gave the mass spectrum shown in Figure 4. It was similar to that of Z32C7, but none of the synthetic standards on hand at the time had the same mass spectrum as P5. A molecular weight of 142 (Figure 4) indicated that the molecule had one double-bond equivalent. Hydrogenation and subsequent GC-MS analysis indicated that a methyl branch was located at the C-3 and confirmed the existence of a double bond. These data, taken together with information from open column chromatography, suggested that the compound was a 3-methyl-heptenoic acid. A mass spectrum of deuterated P5 was similar to that of deuterated Z32C7 and different from that of deuterated (Z)-3-methyl-3-heptenoic acid (Z33C7). The ratios of the mass peak at m/z 60 to the mass peak at m/z 61 were 25% for P5, 38% for Z32C7, and 83% for Z33C7 after hydrogenation with deuterium. The two mass peaks were mostly fragment ions resulting from ω-hydrogen arrangement, and their masses depended on whether

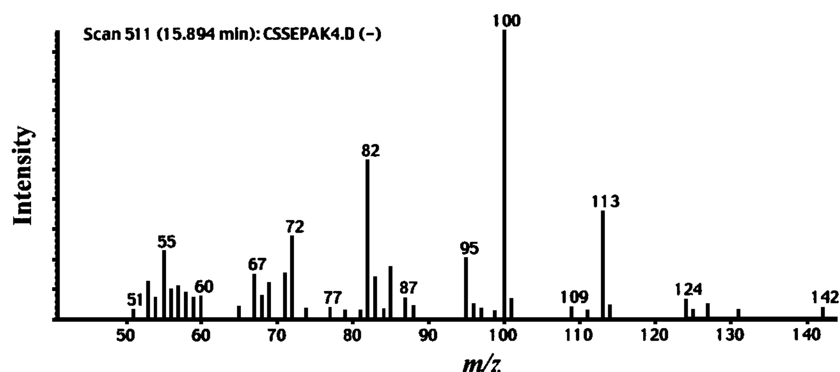

FIG. 4. Mass spectrum of compound P5.

the fragment had 0 or 1 deuterium atom. The base peak was at m/z 61 for all three compounds hydrogenated with deuterium, whereas it was at m/z 60 for all those hydrogenated with hydrogen. The ratio of the mass peaks indicated that two deuterium atoms were at C-2 and C-3 in deuterated P5, suggesting that P5 could be 3-methyl-2-heptenoic acid. GC peaks of the deuterated fatty acids were not totally pure, judging from ion plots and the broad GC peaks compared with those hydrogenated with hydrogen. This finding suggested that the double-bond position determination based on deuteration was not conclusive. Therefore, P5 was identified tentatively as (*E*)-3-methyl-2-heptenoic acid (E32C7). The GC-MS analysis of synthetic E32C7 showed that it had the same mass spectrum as P5 and coeluted with it, confirming the identification. Based on GC data, solvent extracts of one 2-day-old virgin female and C-18-RP column cleanup were estimated to contain 2 ng E32C7 and 2 ng Z32C7.

Fraction 2 contained two GC peaks (Figure 3b). All major GC peaks were identified based on their mass spectra and GC retention times (Table 1), so that male EAG activities to these compounds could be compared to determine if Z32C7 and E32C7 were sex pheromone components. Z32C7 elicited significantly higher male EAG response than did E32C7; both Z32C7 and E32C7 elicited significantly higher male EAG activities than did the control and other fatty acids including hexanoic, heptanoic, octanoic, and nonanoic acids (Figure 5). This implies that Z32C7 and E32C7 are sex pheromone components of *C. subinnotatus*.

Male EAG responses were log-linear to a range of doses of both E32C7 and Z32C7 (Figure 6), indicating that they are physiologically relevant. Male EAG responses to various ratios of E32C7 and Z32C7 were significantly different (Figure 7). The two isomers at equal amounts elicited higher male EAG response than did 90% E32C7 plus 10% Z32C7, 10% E32C7 plus 90% Z32C7, or either

TABLE 1. IDENTIFICATION OF ISOLATED COMPONENTS AS FATTY ACIDS FROM GC-MS DATA

Peak	GC Retention time (min)	MS major fragments (m/z)	Identification
P1	13.28	60 (base), 73, 87	hexanoic acid
P2	14.50	60 (base), 73, 87, 101	heptanoic acid
P3	15.40	55, 67, 69, 72, 82, 95, 100, 113 (base), 142 (M^+)	(Z)-3-methyl-2-heptenoic acid
P4	15.58	60 (base), 73, 84, 101, 115	octanoic acid
P5	15.91	55, 67, 69, 72, 82, 95, 100 (base), 113, 142 (M^+)	(E)-3-methyl-2-heptenoic acid
P6	16.60	57, 60 (base), 73, 87, 98, 115, 129	nonanoic acid
P7	17.60	57, 60 (base), 73, 87, 115, 129, 143, 172 (M^+)	decanoic acid
P8	18.55	55, 60 (base), 73, 87, 98, 115, 129, 143, 186 (M^+)	hendecanoic acid
P9	19.66	57, 60, 73 (base), 85, 101, 115, 129, 143, 157, 171, 200 (M^+)	dodecanoic acid

isomer alone. The male EAG response to 50% E32C7 plus 50% Z32C7 was not significantly different from that to 75% E32C7 plus 25% Z32C7 or that to 25% E32C7 plus 75% Z32C7. The Z32C7 elicited higher male EAG response than did E32C7. Male EAG response to the solvent control was only 0.1 mV (data not shown).

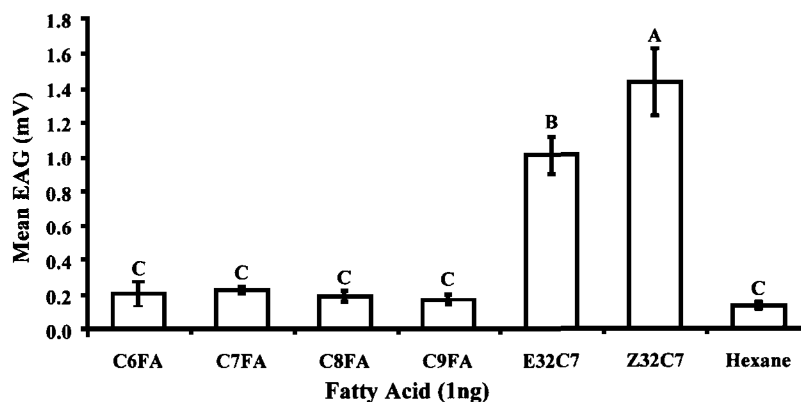

FIG. 5. Male EAG response (mean ± SE) to 1 ng of short-chain fatty acids. C6FA, C7FA, C8FA, C9FA, E32C7, and Z32C7 are hexanoic acid, heptanoic acid, octanoic acid, nonanoic acid, (E)-3-methyl-2-heptenoic acid, and (Z)-3-methyl-2-heptenoic acid, respectively. Hexane is solvent control. Different letters above bars indicate significance (LSD) at $\alpha = 0.05$ ($N = 5$).

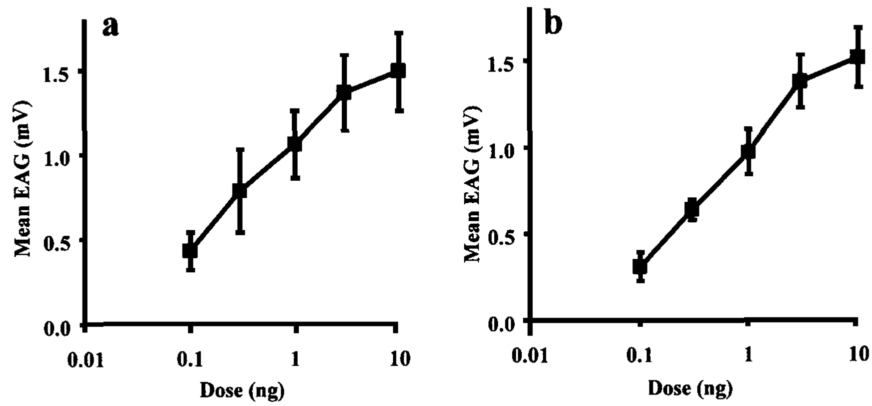

FIG. 6. Male EAG dose responses to (*E*)-3-methyl-2-heptenoic acid (a) and (*Z*)-3-methyl-2-heptenoic acid (b). Note that the scale on *x* axis is logarithmic.

The E32C7 by itself was not attractive to male beetles in Y-tube bioassays, but mixtures with Z32C7 at various ratios, and Z32C7 alone, were attractive compared with control (Figure 8). Very few males failed to enter one of the two arms when a mixture of E32C7 and Z32C7 or Z32C7 alone was tested. In con-

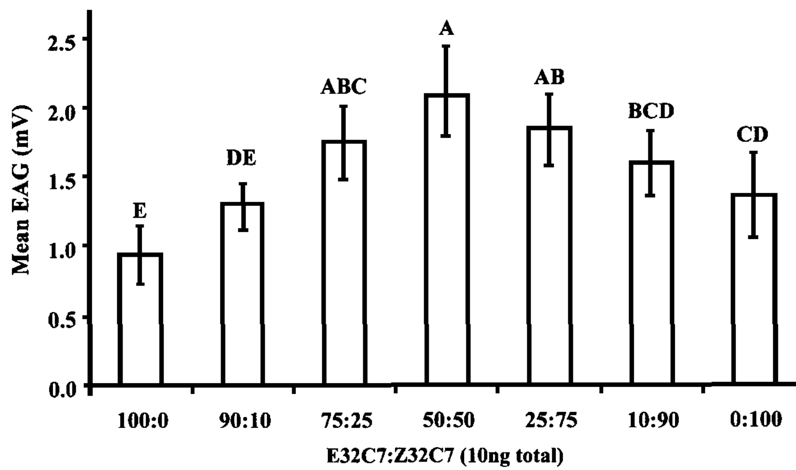

FIG. 7. Male EAG response (mean ± SE) to 10 ng of mixtures of (*E*)-3-methyl-2-heptenoic acid (E32C7) and (*Z*)-3-methyl-2-heptenoic acid (Z32C7) at different proportions. Different letters above bars indicate significance (LSD) at $\alpha = 0.05$ ($N = 5$).

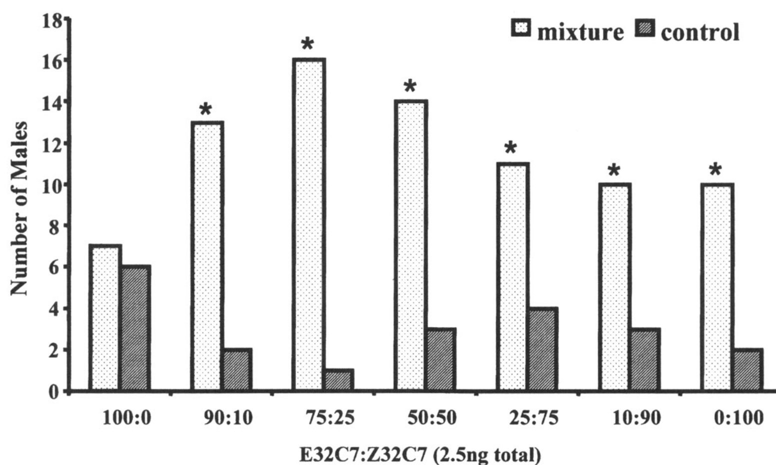

FIG. 8. Male behavior response (number of males choosing to enter one of two arms) to 2.5 ng of mixtures (*E*)-3-methyl-2-heptenoic acid (E32C7) and (*Z*)-3-methyl-2-heptenoic acid (Z32C7) at different proportions versus solvent control. Asterisks above bars of each category indicate significant difference from the expected (response to hexane) (χ^2) at $\alpha = 0.05$ ($N = 20$).

trast, 50% of males did not enter either arm in response to hexane, 25% entered the hexane-treated arm, and the remaining 25% entered the nontreated arm ($N = 40$). When the two isomers were tested individually against three mixtures of different ratios of the two isomers in Y-tube assays, male beetles favored the arm treated with mixtures over that treated with individual isomers (Figure 9), suggesting that mixtures were more attractive than either isomer alone. This implies that both E32C7 and Z32C7 are sex pheromone components of *C. subinnotatus*. However, Z32C7 alone attracted more males than E32C7 alone when both were tested against a mixture. This is in congruence with data in the previous experiment showing that Z32C7 alone could be attractive.

Pitfall-type traps baited with rubber septa treated with 1 ng E32C7 and 1 ng Z32C7 caught more than 80% of the male beetles during scotophase, significantly more than those baited with hexane-treated septa (ca. 20%) (Figure 10). During photophase, pheromone-baited traps caught about 50% of the male beetles, which was significantly higher than were trapped in control traps (ca. 10%). This suggests that both E32C7 and Z32C7 are sex pheromone components of *C. subinnotatus*. Traps treated with the two chemicals trapped significantly higher number of male beetles during the scotophase than during photophase. Earlier studies suggested that male and female *C. subinnotatus* are more likely to mate when they are paired during scotophase than during photophase, and initiation

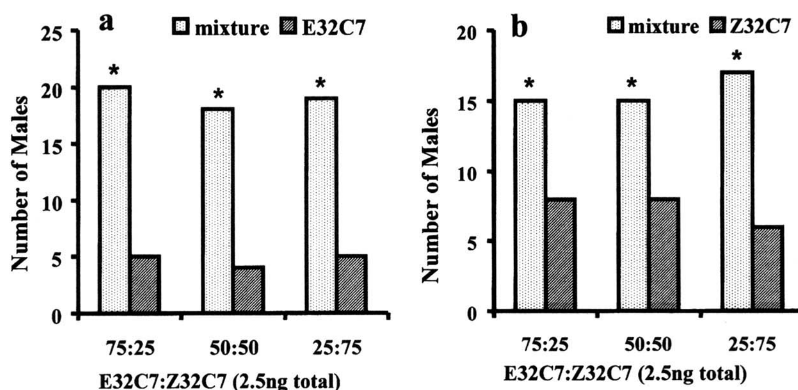

FIG. 9. Male behavior response (number of males choosing to enter one of two arms) to 2.5 ng of mixtures (*E*)-3-methyl-2-heptenoic acid (E32C7) and (*Z*)-3-methyl-2-heptenoic acid (Z32C7) at a different proportion versus E32C7 alone (a) or versus Z32C7 alone (b). Asterisks above bars of each category indicate significant difference from the expected (response to hexane) (χ^2) at $\alpha = 0.05$ ($N = 26$).

of mating takes longer when they are paired in photophase than in scotophase (Mbata et al., 1997). Apparently, *C. subinnotatus* males are more active during nighttime than during daytime in terms of response to sex pheromone and copulation activities.

Sex pheromones have been identified for two other *Callosobruchus* species, *C. analis* (Cork et al., 1991) and *C. maculatus* (Phillips et al., 1996). A copulation releaser, erectin, consisting of a dicarboxylic acid and several hydrocarbons, was identified for *C. chinensis* (Tanaka et al., 1981). Similar to *C. subinnotatus* in this study, both *C. analis* and *C. maculatus* use 3-methyl heptenoic acids as their sexual communication signals. A single compound, (*Z*)-3-methyl-2-heptenoic acid was identified as the sex pheromone in *C. analis* (Cork et al., 1991). Five compounds, four 3-methyl-heptenoic acids and 3-methyleneheptanoic acid, were identified as the sex pheromone of *C. maculatus* (Phillips et al., 1996), although a blend of only two compounds, (*Z*)-3-methyl-3-heptenoic acid and (*Z*)-3-methyl-2-heptenoic acid, was found to be highly attractive to male *C. maculatus* (Shu et al., 1996). The ratio of pheromone components for sexual communication in *Callosobruchus* species may not be as critical as in many Lepidoptera and other insect species. The presence of additional isomers of fatty acids may play a crucial role in differential signaling in *Callosobruchus* species. For example, (*Z*)-3-methyl-2-heptenoic acid alone could be attractive to *C. subinnotatus*, although it is the sex pheromone of *C. analis*. A mixture of two isomers (unique to each species) was more attractive than individual compounds alone in both

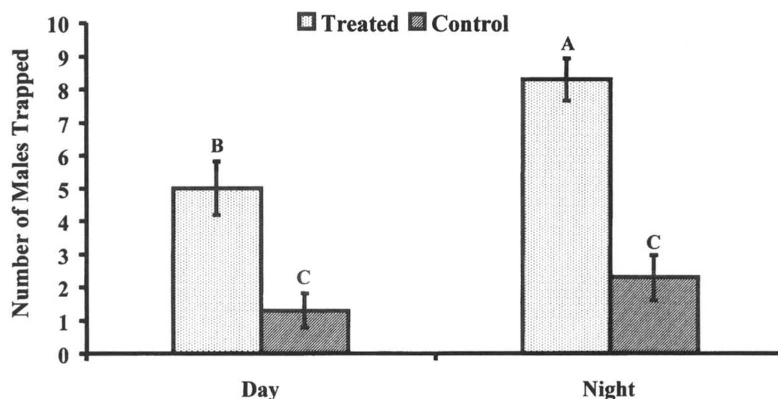

FIG. 10. Male behavior response (number of males out of 10 caught in traps) to a mixture of 1 ng (*E*)-3-methyl-2-heptenoic acid and 1 ng (*Z*)-3-methyl-2-heptenoic acid versus solvent control. Different letters above bars indicate significance (LSD) at $\alpha = 0.05$ ($N = 7$).

C. maculatus (Shu et al., 1996) and *C. subinnotatus*, but males of both species were attracted to a range of ratios of their respective two isomers. In addition to sex pheromone signaling, close-range cues also may be crucial in species recognition.

Further work is needed to verify the attractiveness of E32C7 and Z32C7 to *C. subinnotatus* in storage facilities. Potentially, the sex pheromone identified can be used to manage *C. subinnotatus* in a safer and more environmentally friendly manner by monitoring pest infestation, mass trapping, and/or mating disruption.

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CONTRASTING BEHAVIORAL RESPONSES BY DETRITIVOROUS AND PREDATORY MAYFLIES TO CHEMICALS RELEASED BY INJURED CONSPECIFICS AND THEIR PREDATORS

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Abstract—Larvae of the mayfly *Siphlonisca* are predators of the detritivorous mayfly *Siphlonurus* in floodplain wetlands in Maine (USA). Both mayflies are natural prey of brook trout (*Salvelinus fontinalis*). We exposed larvae of *Siphlonurus* and *Siphlonisca* to chemicals from injured conspecifics and their predators. Significant decreases in movement activity by *Siphlonurus* were elicited by chemicals released from *Siphlonisca*, chemicals released from brook trout fed conspecifics, and by chemicals released from injured conspecifics. A significant decrease in movement activity by *Siphlonisca* was elicited by chemicals released from brook trout fed either conspecifics or *Siphlonurus*. Movement activity by either *Siphlonurus* or *Siphlonisca* was not significantly affected by chemicals released from trout feeding on brine shrimp (*Artemia*). Both *Siphlonurus* and *Siphlonisca* were able to detect chemicals that provided information about past feeding behaviour by brook trout. However, their response to the chemicals used in this study was context-specific. A reduction in movement activity, a behavior that presumably reduces the probability of being consumed by visual predators, occurred only when mayflies were exposed to chemicals released by brook trout feeding on conspecific (*Siphlonurus*) or confamilial (*Siphlonisca*) prey.

Key Words—*Siphlonisca*, *Siphlonurus*, Ephemeroptera, predator avoidance behavior, semiochemicals, alarm pheromones, kairomones, *Salvelinus fontinalis*, wetlands, temporary habitats.

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INTRODUCTION

Modifications of the behavior of freshwater animals in response to chemicals released by their predators or from injured conspecifics have been documented for many taxa, including amphibians, fishes, gastropods, and pelagic and benthic crustaceans and insects (Peckarsky, 1980; Williams and Moore, 1985; Holomuzki and Short, 1988; Dawidowicz et al., 1990; Crowl and Covich, 1990; Covich et al., 1994; Scrimgeour et al., 1994a; Ball and Baker, 1996; Wudkevich et al., 1997; Chivers and Smith, 1998; Kats and Dill, 1998; Van Buskirk and Yurewicz, 1998). When exposed to chemicals released by their predators or from injured conspecifics, benthic insects may show reduced movement activity, sheltering behavior (Peckarsky et al., 1993; Scrimgeour et al., 1994a,b), or increased nocturnal activity relative to daytime activity (McIntosh and Townsend, 1995; McIntosh and Peckarsky, 1996). The chemicals involved have not been identified, but appear to be relatively specific. In some cases prey may react only to predators actively feeding on conspecifics (see review by Chivers and Smith, 1998). In other cases, avoidance behavior is elicited only by suites of cues such as predator odor combined with chemicals released from injured conspecifics (Scrimgeour et al., 1994a). The potential for reaction only to predators that are actively feeding on nearby conspecifics indicates that costs of such avoidance behavior may be reduced by using a relatively fine-tuned mechanism of risk assessment (cf. Ode and Wissinger, 1993).

What are the costs of avoidance behavior? Avoidance behavior usually results in a reduction in time spent feeding, with consequences for rates of growth and development (Feltmate and Williams, 1991; Peckarsky et al., 1993; Ball and Baker, 1996; McPeek and Peckarsky, 1998). Behavior that results in reduced rates of growth may be particularly important for semeloparous insects such as mayflies. Mayflies do not feed as adults, and all somatic tissues allocated for reproduction are determined by the size of the larva upon metamorphosis (McPeek and Peckarsky, 1998). Since fecundity of female mayflies is closely correlated with their size, behavior that results in reduction of growth rate and smaller ultimate size upon emergence may have significant consequences for population demographics and viability (McPeek and Peckarsky, 1998). Nonlethal effects of predator avoidance behavior also will have consequences at the ecosystem level because any reduction in growth or feeding activity will ultimately affect levels of secondary production by prey populations. This in turn will influence production dynamics of adjacent trophic levels (McIntosh and Townsend, 1995, 1996; Peckarsky and McIntosh, 1998).

Because of the potential for interactive effects of multiple predators that operate at multiple trophic levels, consequences of predator avoidance behavior in most food webs will be difficult both to predict and detect (Peckarsky et al., 1997). This scenario becomes even more complex in view of the appar-

ent specificity of predator avoidance behavior with respect to the diet of the predator (e.g., Crowl and Covich, 1990; Mathis and Smith, 1993; Wilson and Lefcort, 1993; Chivers et al., 1996; Mathis and Hoback, 1997; Wisenden et al., 1997). The objective of this study is to use laboratory experiments to examine the behavioral responses of several members of the macroinvertebrate community of a sedge–meadow wetland in eastern Maine (USA) to chemical cues released by their predators. This macroinvertebrate community is unusual in that it is dominated by mayflies of the families Leptophlebiidae (*Leptophlebia*) and Siphlonuridae (*Siphlonurus*, *Siphlonisca*), and because one of these, *Siphlonisca aerodromia* is a predator of the other mayflies (Gibbs and Mingo, 1986; Gibbs and Siebenmann, 1996; Huryn and Gibbs, 1998). Larvae of *Siphlonurus* spp. are largely detritivorous. Larvae of *Siphlonisca* feed primarily on *Siphlonurus* (Gibbs and Mingo, 1986). The brook trout (*Salvelinus fontinalis*), a predator of both *Siphlonurus* and *Siphlonisca*, enters the wetland from adjacent streams to feed during spring floods.

We report the results of laboratory experiments that test four specific predictions: (1) larvae of *Siphlonurus* show predator avoidance behavior (reduced movement activity) in the presence of chemicals released by *Siphlonisca*; (2) larvae of *Siphlonurus* and *Siphlonisca* show predator avoidance behavior in the presence of chemicals released by brook trout actively feeding on conspecifics, (3) larvae of *Siphlonurus* and *Siphlonisca* do not show predator avoidance behavior when in the presence of chemicals produced by brook trout that are not actively feeding on conspecifics, and (4) larvae of *Siphlonurus* and *Siphlonisca* show predator avoidance behavior in the presence of chemicals released by injured conspecifics.

METHODS AND MATERIALS

Study Site and Source of Experimental Animals. The source of mayfly larvae used in this study was a seasonally inundated floodplain north of the confluence of Tomah Stream and Beaver Creek (Washington County, Maine, 45°26′42″N, and 67°34′50″W). Tomah Stream, is a fourth-order tributary of the St. Croix River. During much of the year (June–March), Tomah Stream is confined to its channel. During March–April, melting snowpack causes the river to inundate the floodplain. The inundated area gradually decreases from April to May and the floodplain is generally dry by June. Tussock sedge (*Carex* spp.) is the dominant plant species on the floodplain. Following inundation until the floodplain dries in June, mayflies are abundant within the dense meshwork of sedge detritus that characterizes the habitat. Species of mayflies documented from the Tomah Stream floodplain include the siphlonurids *Siphlonisca aerodromia* and *Siphlonurus mirus*, *S. alternatus*, and *S. quebecensis*, and the

leptophlebiids *Leptophlebia cupida*, *L. nebulosa*, and *L. johnsoni* (Burian and Gibbs, 1991). Together these mayflies contribute 73–94% of the total microinvertebrate biomass on the floodplain (Huryn and Gibbs, 1998). The life history of *Siphlonisca* has been intensively studied (Gibbs and Mingo, 1986; Gibbs and Siebenmann, 1996), and provides a general example of the life cycle of river–floodplain fauna (Huryn and Gibbs, 1998). Larvae first appear beneath the ice of the stream channel during November and remain in the stream until snowmelt during March or April. At this time larvae migrate onto the inundated floodplain. Most larval growth and development occurs here, and adults emerge in late May and early June. After mating flights, females return to the stream and oviposit. Eggs hatch in the stream the following November. The life cycles of the dominant species of *Siphlonurus* from the Tomah floodplain follow a similar pattern (Huryn and Gibbs, 1998).

Siphlonisca is the major macroinvertebrate predator on the floodplain during inundation, where it contributes 80% to total biomass of predacious invertebrates (Huryn and Gibbs, 1998). The most common prey are larvae of *Siphlonurus*, but *Leptophlebia*, *Eurylophella* (Ephemeroptera: Ephemerellidae), and midge larvae are also consumed (Gibbs and Mingo, 1986). *Siphlonurus* is primarily detritivorus and herbivorous, but may consume animal material in later instars. The relative importance of animal prey compared to biofilm and organic particles is unknown (Edmunds et al., 1976). Vertebrates that prey on aquatic macroinvertebrates are conspicuous during floodplain inundation at Tomah Stream. The common shiner (*Notropis cornutus*), three-spine stickleback (*Gasterosteus aculeatus*), chain pickerel (*Esox niger*), common white sucker (*Catostomus commersoni*), and brook trout all have been reported from the floodplain during inundation. While on the floodplain, these fish feed heavily on macroinvertebrates, particularly mayflies (Gibbs and Mingo, 1986). Their quantitative and qualitative effects on the floodplain macroinvertebrate fauna, however, are unknown.

Experimental Animals. Larvae of *Siphlonurus* and *Siphlonisca* and conditioned sedge detritus were collected by dip net on April 29 and May 14, 1998. Larvae were placed in plastic bags containing water and stored on ice during transport. In the laboratory, larvae and sedge detritus were placed in 37-liter glass aquaria containing 25-liters of aerated well water (water temperature: 17°C, light–dark cycle 14 : 10 hr). Three aquaria each were used to house *Siphlonurus* and *Siphlonisca*. *Siphlonurus* were fed ad libitum on the biofilm associated with sedge detritus. *Siphlonisca* fed upon *Siphlonurus* larvae that were continually provided as a source of prey. Except for several individuals that died while molting and instances of cannibalism by *Siphlonisca*, mortality did not appear to be significant. Larval growth during the experiments was readily apparent as an increase in size by both taxa. Brook trout were collected from a stream in central Maine. One brook trout was placed in each of three separate 37-liter glass aquaria containing 25-liters of well water and was fed either *Siphlonurus*,

Siphlonisca, or brine shrimp (*Artemia*) for the duration of the experiments (April 29–May 18, 1998). For logistical reasons and because of the low variability in semiochemicals produced by members of a given fish population (Mathis et al., 1993; Brown et al., 1995), single fish were used for each predator–prey combination. The trout were selected so that they were as close to the same size as practicable. The total lengths of all fish used in the experiments ranged from 126 to 133 mm.

Preparation of Stimuli. Eight stimuli were produced: (1) brook trout fed *Siphlonurus*, (2) brook trout fed *Siphlonisca*, (3) brook trout fed brine shrimp, (4) *Siphlonisca* fed *Siphlonurus*, (5) *Siphlonisca* fed *Siphlonurus* + injured *Siphlonurus* chemicals, (6) injured *Siphlonurus*, (7) injured *Siphlonisca*, and (8) unadulterated well water (base stimulus or control).

Trout-based stimuli were prepared by the following protocol. Trout were allowed to feed ad libitum on appropriate diets (e.g., *Siphlonurus*, *Siphlonisca*, brine shrimp) from April 29–May 18, 1998, except for 24-hr periods prior to experiments. At the beginning of this 24-hr period, tanks were cleaned and drained and the water was replaced with aerated well water. No additional prey were introduced during this period. At the end of this 24-hr period, water containing chemicals released by the fish was removed from the tanks and immediately used as a stimulus. The *Siphlonisca* stimulus was prepared by placing four individuals, without prey, in a 300-ml glass bowl containing ~200 ml of well water for a 24-hr period prior to experiments. A stimulus combining *Siphlonisca* chemicals with injured *Siphlonurus* chemicals was also prepared by placing 16 *Siphlonurus* and 4 *Siphlonisca* in a 300-ml glass bowl containing ~200 ml of well water for 24 hr before experiments. On each occasion all *Siphlonurus* were consumed prior to the experiment. Stimuli from injured mayflies were prepared by grinding either two *Siphlonurus* or one *Siphlonisca* larvae in 10 ml of well water using a mortar and pestle. The resulting solutions were filtered through a fine mesh net. All stimuli were used immediately following preparation.

Behavioral Assays. Experiments were performed on May 11, 12, and 18, 1998, during daylight hours using 300-ml circular (10.5-cm-ID) glass dishes containing ~200 ml of aerated well water maintained at room temperature. For experiments using *Siphlonurus*, five larvae were arbitrarily selected and placed in the dishes (10 dishes were used at a time) and were allowed to acclimate for 5 min. After the acclimation period, each dish was observed at 15-sec intervals and the instantaneous number of individuals swimming was recorded. After 5 min, 5 ml of stimulus was added to the center of each dish using a polyethylene syringe, and the observation procedure was repeated for another 5 min. This resulted in 20 prestimulus and 20 poststimulus observations. Stimuli were randomly assigned to the 10 dishes used in each trial. This procedure was repeated until 20 replicates were obtained for each treatment. Individual larvae were used only once. The mean ± SD total length (excluding cerci) of a sample of 40 test animals was

8.2 ± 1.3 mm. The treatments consisted of: (1) brook trout fed *Siphlonurus*, (2) brook trout fed *Siphlonisca*, (3) brook trout fed brine shrimp, (4) *Siphlonisca* fed *Siphlonurus*, (5) *Siphlonisca* fed *Siphlonurus* + injured *Siphlonurus* chemicals, (6) injured *Siphlonurus*, and (7) well water (control).

A similar protocol was followed by *Siphlonisca*, except that only one larva was placed in the observation dishes. The treatments consisted of: (1) brook trout fed *Siphlonurus*, (2) brook trout fed *Siphlonisca*, (3) brook trout fed brine shrimp, (4) injured *Siphlonurus*, (5) injured *Siphlonisca*, and (6) well water (control). Along with these treatments, a seventh treatment consisting of a mixture of brook trout fed brine shrimp (5 ml) and injured *Siphlonisca* (5 ml), was used as a stimulus. Twenty replicates were obtained for each treatment, except the latter where only 18 replicates were obtained because of limited numbers of *Siphlonisca* larvae. The number of replicates was limited to ≤20 because of constraints in the number of *Siphlonisca* we had legal permission to collect. *Siphlonisca* is protected by the state of Maine (McCollough, 1997). The mean ± SD total length (excluding cerci) of a sample of 11 test animals was 17.1 ± 1.8 mm.

Analysis. For each replicate, the difference of the total number of individuals moving during 20 scans (*Siphlonurus*), or the number of scans recording movement (*Siphlonisca*), between the pre- and poststimulus periods was calculated. Positive values indicated increased movement activity following addition of a stimulus; negative values indicated decreased activity. Means of each treatment were compared with the control using a one-tail t test. The family-wise error rate was assessed and controlled using the modified Bonferroni test following Keppel (1982). Assuming that comparisons are orthogonal and are based on specific a priori predictions, the modified Bonferroni test specifies that corrections to the family-wise error rate be introduced only when the number of comparisons exceeds $k-1$, where k is the number of treatments (Keppel, 1982). In this study there were a total of seven treatments (six experimental treatments and one control) for each set of experiments (e.g., *Siphlonisca* is the first set, *Siphlonurus* the second set). Since the analysis was restricted to six preplanned orthogonal comparisons that were based on specific a priori predictions for each set of experiments, the rejection probability (P) was set at 0.05 for each comparison (Keppel, 1982).

RESULTS

There was a significant reduction of movement activity by *Siphlonurus* when exposed to chemicals released by *Siphlonisca* ($t = -2.5893$, $df = 38$, $P = 0.007$, one-tail t test; Figure 1). Both *Siphlonurus* ($t = -3.1051$, $df = 38$, $P = 0.002$, one-tail t test; Figure 1) and *Siphlonisca* ($t = -2.5152$, $df = 38$, $P = 0.008$, one-tail t test; Figure 2) showed significant decreases in movement behav-

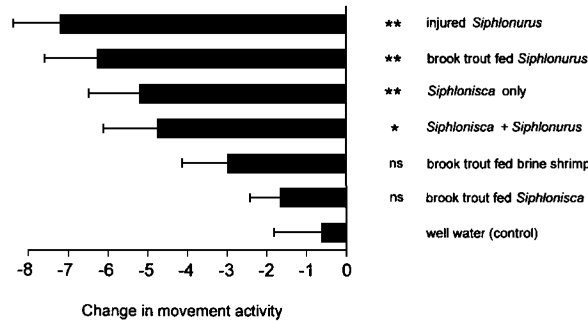

FIG. 1. Graph showing response of *Siphlonurus* larvae to chemical cues from injured conspecifics and predators: the mayfly *Siphlonisca* and brook trout fed various diets. The x axis indicates change in the average number of observations of the number of individuals swimming out of groups of five before and after being exposed to a chemical stimulus (see text for details). Stimuli are listed on the Y axis. ns = mean not significantly different from control, $^{*}P < 0.05$, $^{**}P < 0.01$. Error bars are standard errors ($N = 20$).

ior when exposed to stimuli containing chemicals released by brook trout feeding on conspecifics when compared to the control.

When exposed to chemicals released by trout feeding on prey other than conspecifics, however, differences in movement activity of larvae of *Siphlonurus* were not significantly different from controls. For example, the reduction

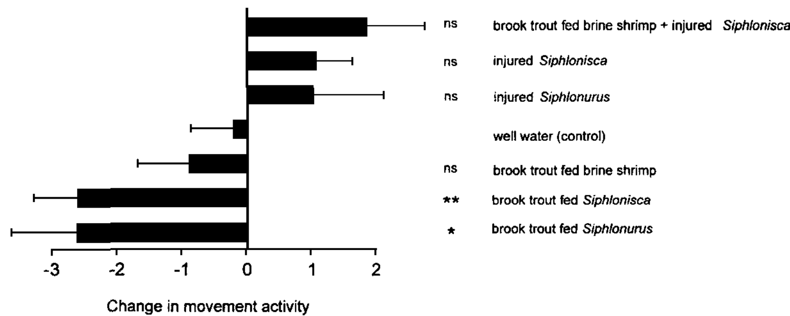

FIG. 2. Graph showing response of *Siphlonisca* larvae to chemical cues from injured conspecifics, injured *Siphlonurus* (prey) and brook trout fed various diets (predator). The x axis indicates change in the average number of observations of single individuals moving before and after being exposed to a chemical stimulus (see text for details). Stimuli are listed on the y axis. ns = mean not significantly different from control, $^{*}P < 0.05$, $^{**}P < 0.01$. Error bars are standard errors ($N = 20$ except for "trout fed brine shrimp + injured *Siphlonisca*" where $N = 18$).

of movement activity by *Siphlonurus* when exposed to chemicals released by brook trout fed either brine shrimp (t = −1.4046, df = 38, P = 0.084, one-tail t test; Figure 1) or brook trout fed *Siphlonisca* (t = −0.7249, df = 38, P = 0.236, one-tail t test; Figure 1) was not significantly different from the control. In conrast to *Siphlonurus*, movement activity of *Siphlonisca* was significantly reduced following exposure to chemicals released by brook trout feeding on either conspecifics (t = −2.5152, df = 38, P = 0.008, one-tail t test, Figure 2) or *Siphlonurus* (t = −1.9494, df = 38, P = 0.029, one-tail t test; Figure 2). However, the reduction of movement activity by *Siphlonisca* exposed to chemicals from brook trout fed brine shrimp was not significantly different from the control (t = −0.6715, df = 38, P = 0.253, one-tail t test; Figure 2). When exposed to chemicals released by injured conspecifics, *Siphlonurus* showed a highly significant reduction in movement activity when compared with controls (t = −3.7784, df = 38, $P < 0.001$, one-tail t test; Figure 1). In comparison, movement activity by *Siphlonisca* did not decrease significantly in the presence of chemicals released by injured conspecifics (t = 1.4065, df = 38, P = 0.916, one-tail t test; Figure 2). It should also be noted that all treatments involving brook trout chemicals without the presence of chemicals released directly from injured mayflies, either conspecifics or *Siphlonurus*, elicited a trend toward decreased movement activity by *Siphlonisca*, and that all treatments involving the presence of chemicals released directly by injured mayflies elicited a trend toward increased movement activity by *Siphlonisca* (Figure 2).

DISCUSSION

The siphlonurid mayflies *Siphlonisca* and *Siphlonurus* showed contrasting behavioral responses to chemicals released by injured conspecifics and their predators. The detritivorous mayfly *Siphlonurus* showed a significant decrease in movement activity when exposed to chemicals released by injured conspecifics, as has been shown for other mayflies (e.g., *Baetis*; Scrimgeour et al., 1994a). The response by the predacious mayfly *Siphlonisca*, however, was exactly the opposite of *Siphlonurus*, its primary prey. *Siphlonisca* increased movement activity when exposed to chemicals released by injured conspecifics and *Siphlonurus*. The apparent trend toward increases in activity were consistent among all treatments involving chemicals from injured mayflies. Such a behavioral response was not anticipated, contrasted strongly with results from other studies of mayfly antipredator behavior (Scrimgeour et al., 1994a,b; Peckarsky et al., 1993; McIntosh and Peckarsky, 1996; McIntosh and Townsend, 1995), and deserves further interpretation in the context of the specific biology and habitat of *Siphlonisca*.

Increased activity by *Siphlonisca* in response to chemicals released by

injured mayflies may enhance detection of injured or freshly killed prey nearby. Field and laboratory observations, indicate that *Siphlonisca* will feed opportunistically on dead animal tissue (e.g., exuviae; A. D. Huryn, personal observation) and is cannibalistic. Chemicals released by injured or freshly killed invertebrates, including conspecifics, may thus elicit a search response by *Siphlonisca* that is similar to those reported for spiny lobsters in marine habitats (Zimmer-Faust, 1993) and predacious stonefly larvae in streams (Dodson et al., 1994). The detection of chemical signals may be particularly important for inhabitants of sedge-meadow wetlands because the dense meshwork provided by sedge detritus obscures clear fields of vision beyond more than a few centimeters.

In comparison to their contrasting responses to injured conspecifics, the responses of *Siphlonurus* and *Siphlonisca* to chemicals produced by brook trout were similar and highly context specific. Both *Siphlonurus* and *Siphlonisca* modified their movement activity in response to chemicals released from brook trout. Furthermore, both mayflies were able to use information from these chemicals to assess past feeding behavior by brook trout. *Siphlonurus* showed significant reductions of movement activity in response to chemicals released by brook trout only in cases when the trout had fed upon conspecifics. In comparison, *Siphlonisca* showed a significant reduction in movement activity when exposed to chemicals released by brook trout fed either conspecifics or *Siphlonurus*. Although the response of *Siphlonisca* to chemicals released by brook trout was more general than that of *Siphlonurus* (e.g., responds to brook trout feeding upon confamilial rather than only conspecific mayflies), neither taxon showed a significant reduction of movement when exposed to chemicals released by brook trout feeding on brine shrimp.

The more general (confamilial) response of *Siphlonisca* compared with the more precise (conspecific) response of *Siphlonurus* seems to make good evolutionary sense, given the ecology of the two mayflies. First *Siphlonisca* is a rare mayfly with a highly localized distribution, being known from fewer than 20 locations worldwide (e.g., McCollough, 1997), whereas *Siphlonurus* is widespread throughout much of the Holarctic (Edmunds et al., 1977). Second as expected for a predator, *Siphlonisca* is much less abundant than its primary prey, *Siphlonurus*, wherever these taxa occur together. For example, benthic samples taken from the study site on May 14, 1998, indicated that the mean abundance of *Siphlonisca* was 148 ± 56 individuals/m^2 (± SE, $N = 5$), whereas the mean abundance of *Siphlonurus* was 4541 ± 875 individuals/m^2 (A. D. Huryn, unpublished). Third both mayfly taxa occupy similar habitats, are active swimmers, and presumably are similarly attractive to foraging trout. Assuming that the risk of either *Siphlonurus* or *Siphlonisca* being detected by a trout is proportional to their relative abundances, and that the risk of being consumed by a trout following detection is similar between taxa, it seems that a response by *Siphlonisca* to chemicals produced by predators feeding upon *Siphlonurus* would be adaptive.

Siphlonisca may simply be too rare in terms of distribution and abundance to elicit a similar response from *Siphlonurus*.

Compared with *Siphlonurus*, the response of *Siphlonisca* to the various stimuli used in the experiments was complex, and indicated the conflicting demands of efficient and simultaneous detection of both predators and prey. The importance of the pattern of response observed in this study becomes particularly clear when considered in the context of the ephemeral floodplain habitat of *Siphlonisca*. It has been shown that levels of hunger will influence the amount of risk assumed by *Baetis* mayfly larvae in streams (Scrimgeour et al., 1994b). Because *Siphlonisca* inhabits a temporary wetland for a critical period of its life history (Gibbs and Mingo, 1986; Huryn and Gibbs, 1998), risk of mortality from desiccation rather than hunger may have the greatest consequences for fitness. *Siphlonisca* completes ~95% of its growth as biomass during a short (two month) period of floodplain inundation during the spring (A. D. Huryn, personal observation). If growth and development are not completed relatively quickly, larvae risk mortality from stranding as the floodplain dries. The risk of stranding and desiccation before completing development also has bearing on the nonlethal consequences of predator avoidance behavior by temporary-wetland species of *Siphlonurus* as well. This balance of conflicting risks raises interesting questions about the more subtle nonlethal effects of avoidance behavior on growth and the immediate risk of mortality by predation on the life history strategies of aquatic macroinvertebrates in temporary environments (Crowl, 1990).

The adaptive significance of the reduction of movement behavior by *Siphlonurus* and *Siphlonisca* in response to chemicals signaling the presence of a visual predator is presumably to reduce mortality by direct predation. It is probable that nonlethal effects of this behavior will result in reduced food intake, growth, and probably fecundity of adults (McPeek and Peckarsky, 1998). This response is also apparently tuned to specific chemicals released by specific predators, as shown for damselfly larvae by Chivers et al. (1996). In this study, significant reductions in movement activity of both *Siphlonurus* and *Siphlonisca* in response to brook trout chemicals were observed only for treatments that included an element of direct and active predation on conspecific or confamilial mayflies. This indicates that these mayflies may be able to reduce the nonlethal effects of predator avoidance behavior by detecting active predation on mayflies in their vicinity, rather than the simple presence of a potential predator.

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TERPENE ATTRACTANT CANDIDATES OF *Dioryctria sylvestrella* IN MARITIME PINE (*Pinus pinaster*) OLEORESIN, NEEDLES, LIBER, AND HEADSPACE SAMPLES

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Abstract—Capillary GC analysis was used to determine the proportion and quantity of terpenes in wood resin, pentane extracts from needles and liber, and headspace samples of needles and pruning wounds in 24 thirteen-year-old maritime pine. Fifteen different terpenes were identified in the samples. Germacrene D and β-pinene were the dominant terpenic compounds in the needles, while α-pinene and β-pinene were dominant in the liber and wood resin. Headspace samples of both needles and pruning wounds contained essentially monoterpenes. Only trace amounts of sesquiterpenes were found in pruning wound emissions. The presence of an oxygenated compound, linalool, in the pruning wound emissions is discussed, although this compound is not found in Maritime pine essential oil. Twelve of the 24 trees studied were infested by *Dioryctria sylvestrella*. Maritime pine susceptibility to this insect was related to the terpene composition of the different samples.

Key Words—*Pinus pinaster*, wood resin, needle, liber, headspace, terpenes, insect-plant interaction, *Dioryctria sylvestrella*.

INTRODUCTION

Terpenes are a major volatile component of coniferous resin and have often been studied because of their multiple ecological roles (Harborne, 1990; Langenheim, 1994). They affect the behavior of a variety of insects (reviewed by Speight and Wainhouse, 1989; Honda, 1995) and may act as attractants, oviposition stimulants, or defenses. Monoterpenes and sesquiterpenes have also been

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used as biochemical markers in forest genetics because many of these compounds have been shown to be under strong genetic control and are little influenced by environmental factors (Baradat et al., 1991; Hanover, 1992).

Terpenes have been shown to affect the behavior of *Dioryctria* species such as *D. amatella* (Hulst) (Fatzinger and Merkel, 1985; Hanula et al., 1985), *D. horneana* (Dyar) (Valterova et al., 1995), and *D. abietivorella* (Grote) (Shu et al., 1997). The European stem borer, *D. sylvestrella*, is a major pest of maritime pine plantations in Aquitaine, a region of France that represents the largest unit of artificial forest in Europe (1.2 million ha). This oligophagous insect is restricted to the genus *Pinus* in Eurasia (Menassieu et al., 1989) with occasional damage to *Picea* spp. (Menassieu, unpublished observations). Unlike other *Dioryctria* spp., *D. sylvestrella* does not damage cones or terminal shoots; the moth attacks primarily the main stem and occasionally the branches. In southwest France, adult moths emerge from mid-June to July and, after mating, eggs are deposited approximately 10 days later (Menassieu et al., 1989). Eggs are laid individually on the trunk under bark scales and hatch in 15–20 days (Zocchi, 1961). Larvae tunnel through the bark into the phloem where they feed until October. The larvae overwinter in their gallery and recommence boring activity in March until the pupal stage (May–June). Adult moths emerge approximately 21–30 days after pupation and have a life-span of 15–21 days (Menassieu et al., 1989). If larvae succeed in girdling the stem, the tree may suffer from interruption of sap flow, resulting in a decrease of the annual increment or in a malformation of the trunk and in increasing the risk of windbreak (Baronio and Butturini, 1988).

Previous studies (Jactel et al., 1994, 1996a; Jactel and Kleinhentz, 1997) have demonstrated that volatiles exuding from pruning wounds or bark cracks may attract *D. sylvestrella* to host trees. Jactel et al. (1996b) related maritime pine susceptibility to *D. sylvestrella* to the proportion of some monoterpenes in the wood resin. Infested trees should exhibit either cracked bark or a high percentage of terpinolene in the wood resin to release the requisite amount of attractant into the atmosphere (Kleinhentz et al., 1998).

Wood resin composition appears to play an important role in host selection by *D. sylvestrella*. However, resin ducts are found throughout the maritime pine tree and are located within the parenchyme of buds, leaves, and cortex and in phloem and xylem (Pauly, 1962; Baradat and Marpeau-Bezard, 1988). Within the tree, the nature and relative amount of these components varies within organs or tissues (Bernard-Dagan, 1968; Pauly et al., 1973). Variability in terpene composition also exists between trees (Bernard-Dagan et al., 1971; Baradat et al., 1978) due to some physiological and genetic properties that control terpene biosynthesis (reviewed by Baradat et al., 1991). Moreover, terpene emission rates do not necessarily reflect abundance within the tissue. Environmental factors (light, temperature, humidity) also influence the emission rate (Rasmussen, 1972; Yokouchi and Ambe, 1984; Evans et al., 1985).

The high cost of the intensive management of this maritime pine forest makes it necessary to prevent stem borers from reducing the volume and the quality of the forest products grown. The impossibility of chemical control, because the insect is hidden under the bark for more than 11 months each year and the uncertainty of biological controls, led us to investigate other pest management strategies, including the use of host chemicals as insect attractants. Thus, the goal of this study was to identify candidate terpenes that could be attractants to *D. sylvestrella*. For this, the terpene compositions of resin from different tissues (wood, needle and liber) and the terpene emission generating by pruning wounds and needles were determined.

METHODS AND MATERIALS

The study was conducted in maritime pine from a local provenance grown on a mesophilous humid heath at the Station de Recherches Forestières, Gazinet, France. Trees were 13 years old; 12 infested trees (mean diameter at breast height = 16.6 ± 2.6 cm) and 12 uninfested trees (mean diameter at breast height = 15.9 ± 1.4 cm) were randomly selected from, respectively, the 44 infested and 183 uninfested trees initially identified for the study. All samples were taken during the period of *D. sylvestrella* flight (from June to August).

Resin Samples. Wood resin was collected 2 m above ground level using methods described by Jactel et al. (1996b) and oleoresin samples were stored at −30°C under nitrogen until analyses were performed (Delorme and Lieutier, 1990). In addition, 5 g of needles (1 and 2 years old) and 5 g of liber were collected at the 5-year-old internode of each tree. Resins were extracted in pentane for 24 hr. The extracts were filtered through a 70–230 mesh (10 ml) column of SDS silica gel. Mono- and sesquiterpenes were eluted with 30 ml of pentane–ether (98 : 2). The purified extracts were concentrated by evaporation under moderate vacuum before FID-GC analyses.

Headspace Samples. Two different headspace techniques were developed to collect terpene emissions from needles and pruning wounds. The first trap had been designed for needle emission collection in the laboratory and was adapted by Roques (personal communication). Needles (50 g) were enclosed in a Tedlar bag perforated with air inlet holes and connected by a Teflon tube to a glass cartridge filled with 100 mg XAD_2. The trap was connected by Teflon tubes to a vacuum pump (90 ml/min). Eight samples of needles of different trees (infested or not), including a blank control, were analyzed simultaneously. The effluvial sampling was run for 15 hr. The adsorbent was eluted using 400 μl dichloromethane (CH_2Cl_2). The estimated trapping efficiency for XAD_2 and CH_2Cl_2 use is between 47 and 78% for low-molecular-weight compounds such as terpenes (Figure 1), using the method described by Mathieu (1995).

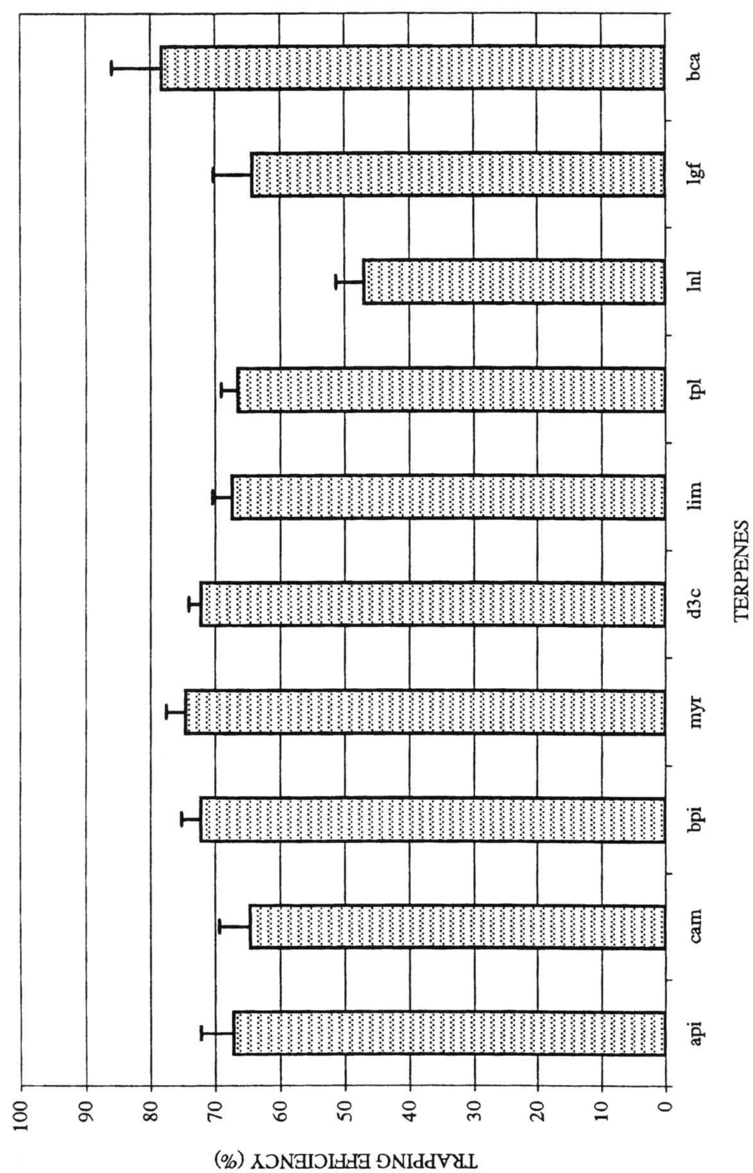

FIG. 1. Trapping efficienty for some terpenes using XAD2 and dichloromethane for adsorption and desorption cycles. api: α-pinene, cam: camphene, bpi: β-pinene, myr: myrcene, lim: limonene, tpl: terpinolene, lnl: linalool, lgf: longifolene, bca: β-caryophylene.

Since the infestation rate for *D. sylvestrella* increases with pruning severity (Jactel et al., 1994, 1996a; Jactel and Kleinhentz, 1997), a second trap was designed to collect volatiles emitted by pruning wounds from a living tree. The sampling apparatus consisted of two polytetrafluoroethylene (PTFE) chambers (500 ml) with an open rectangular shaped side (4 × 10 cm). The first chamber was attached by wire to the trunk around a pruning wound. The second chamber was attached to a glass plate (control chamber). The edge of the chamber, which touched the tree or the glass plate was wrapped with PTFE to prevent gas exchange between the chamber and the atmosphere. Incoming air was filtered using an XAD_{16} cartridge. The chamber was also connected to a glass cartridge filled with 100 mg XAD_2, by a Teflon tube. The cartridge was finally connected to a vacuum pump (180 ml/min) by Teflon tubes. The effluvial sampling was run for 48 hours. The adsorbent was eluted using 400 μl CH_2Cl_2.

FID-GC Analysis. The purified extract was analyzed by FID-GC (Hewlett Packard 5890 series II; temperature program: 60°C to 90°C at a rate of 6°C/min, 90°C to 102°C at a rate of 3°C/min, 5 min at 102°C, and 102°C to 280°C at a rate of 6°C/min; splitless injector 270°C; detector 290°C) on a 30 × 0.25 mm ID HP-1 column (He 15 psi, 2.5-μl sample). The relative percentage of mono- and sesquiterpenes was calculated by adding up all recorded terpene peaks. Components were also quantified by comparing their peak area with that of an *n*-dodecane standard.

Statistical Analyses. All statistical analyses were performed using SAS software (SAS Institute, 1996). Statistical analyses of percentage variables were computed using the arcsin $\sqrt{x}$ transformation (Dagnelie, 1973). Nonparametric analyses of variance (Wilcoxon test) were used for oleoresin composition data that were proportions of *p*-terpenes with the constraint $\Sigma_{pi} = 1$. Correlation between terpenes was estimated using the Pearson correlation coefficient. Mean differences were tested using Scheffe's multiple range test.

RESULTS AND DISCUSSION

Fifteen terpenes or oxygenated compounds exhibited relative proportions higher than 0.1% in the different tree tissues and emissions. In order of increasing retention times, there were seven monoterpenes (α-pinene, camphene, β-pinene, myrcene, δ-3-carene, limonene, terpinolene), an oxygenated monoterpene (linalool) and seven sesquiterpenes (longipinene, α-copaene, α-cubebene, longifolene, β-caryophyllene, α-humulene and germacrene D).

Within-Tree Variation in Terpene Composition of Maritime Pine Resin

The terpene compositions of resin from the different tissues (wood, needle, and liber) showed some qualitative differences. For example, longipinene and longifolene were absent from the needle resin. In addition, δ-3-carene and α-copaene were absent from the liber resin (Table 1).

TABLE 1. VARIATION IN MARITIME PINE TERPENE PROPORTIONS (%) IN WOOD, NEEDLE, AND LIBER RESIN AND IN NEEDLE AND PRUNING WOUND EMISSIONS[a]

Terpenes	Sample effect (F value)	Percent (mean ± SE)				
		Wood resin	Liber resin	Needle resin	Needle emission	Pruning wound emission
α-Pinene	184.16^{b}	58.52 ± 1.80^{ab}	52.55 ± 1.20^{b}	17.01 ± 0.59^{d}	32.69 ± 1.48^{c}	63.52 ± 1.76^{a}
Camphene	68.20^{b}	0.56 ± 0.01^{b}	0.95 ± 0.07^{a}	0.18 ± 0.01^{c}	0.48 ± 0.03^{b}	0.58 ± 0.01^{b}
β-Pinene	14.32^{b}	31.86 ± 1.77^{b}	28.32 ± 1.51^{b}	28.69 ± 1.27^{b}	43.00 ± 1.69^{a}	32.68 ± 1.57^{b}
Myrcene	15.06^{b}	1.17 ± 0.02^{b}	1.34 ± 0.20^{b}	2.99 ± 0.49^{b}	5.77 ± 0.94^{a}	1.57 ± 0.27^{b}
δ3-Carene	47.68^{b}	0.02 ± 0.00^{c}	0^{c}	4.30 ± 0.61^{b}	10.40 ± 1.34^{a}	0.02 ± 0.01^{c}
Limonene	76.26^{b}	2.03 ± 0.04^{c}	2.43 ± 0.12^{c}	4.72 ± 0.25^{b}	7.06 ± 0.45^{a}	1.36 ± 0.28^{c}
Terpinolene	9.73^{b}	0.33 ± 0.07^{b}	2.12 ± 0.56^{a}	0.63 ± 0.11^{b}	0.61 ± 0.09^{b}	0.03 ± 0.01^{b}
Linalool	134.91^{b}	0^{b}	0^{b}	0^{b}	0^{b}	0.17 ± 0.01^{a}
Longipinene	6.02^{b}	0.06 ± 0.02^{a}	0.01 ± 0.01^{b}	0^{b}	0^{b}	0^{b}
α-Copaene	120.20^{b}	0.01 ± 0.00^{b}	0^{b}	0.13 ± 0.01^{a}	0^{b}	0^{b}
α-Cubebene	157.92^{b}	0.11 ± 0.01^{b}	0.01 ± 0.01^{bc}	0.67 ± 0.05^{a}	0^{c}	0^{c}
Longifolene	5.58^{b}	0.71 ± 0.27^{ab}	1.70 ± 0.65^{a}	0^{b}	0^{b}	0.05 ± 0.00^{b}
β-Caryophyllene	118.21^{b}	4.04 ± 0.24^{c}	7.47 ± 0.71^{b}	10.90 ± 0.63^{a}	0^{d}	0.03 ± 0.01^{d}
α-Humulene	54.71^{b}	0.57 ± 0.03^{b}	2.06 ± 0.28^{a}	1.68 ± 0.07^{a}	0^{b}	0^{b}
Germacrene D	589.05^{b}	0.02 ± 0.00^{b}	1.05 ± 0.12^{b}	28.08 ± 1.14^{a}	0^{b}	0^{b}

[a]Five samples; $df = 4$. Means with the same letter are not significantly different (Scheffe's multiple range test, $\alpha = 0.05$) within rows.
[b]$P < 0.001$.

Few changes in the relative proportion of terpenes were recorded between the wood and liber resins (Table 1). α-Pinene and β-pinene were the dominant terpenic compounds in these two tissues, averaging 80% and 90% of the total amounts of terpenes in the liber and wood resin, respectively. The terpene composition of these two resins was consistent with that found in the literature for maritime pine liber resin (Bernard-Dagan, 1968) and wood resin (Jactel et al., 1996b).

Needle resin composition was very different from the terpene profile of the wood and liber resin (Table 1). α-Pinene and β-pinene averaged only 45% of the total terpene amounts. Sesquiterpenes, which represented less than 5% in the wood resin, averaged 45% in the needle resin. Germacrene D and β-pinene were the dominant terpenic compounds in the needles. The terpene composition of the needle resin was very similar to that demonstrated by Pauly et al. (1973).

This study has confirmed the existence of two resins that have different terpene proportions. Baradat and Marpeau-Bezard (1988) also ranked maritime pine resins into two categories. The primary resin, synthesized in primary tissues such as the needles, is characterized by an equal proportion of mono- and sesquiterpenes. The secondary resin, synthesized in secondary tissues such as the liber and wood, is characterized by a high proportion of α-pinene and β-pinene (between 85% and 95% of total terpenes).

Tree diameter was not correlated with terpene proportion in the three resin profiles. Kleinhentz et al. (1998) have also shown a lack of any correlation between tree diameter and terpene proportion in the wood resin. Pinaceae oleoresins generally consist of 50–90% nonvolatile compounds (essentially diterpenes) and 10–50% volatile mono- and sesquiterpenes (Kramer and Kozlowski, 1979). In maritime pine, the terpene quantity in the wood resin averaged 160 mg/g resin (Table 2). Large differences existed between the different tissues. These differences in terpene quantities resulted from differences in terpene biosynthesis as well as from anatomical differences such as the number of resin ducts and duct diameter (Baradat and Marpeau-Bezard, 1988). The quantity of mono- and sesquiterpenes averaged 13 mg/g fresh needles, while it averaged only 3 mg/g of fresh liber (Table 2). Baradat and Marpeau-Bezard (1988) have also shown that primary tissues such as needles have a higher capacity for terpene synthesis than secondary tissues such as liber and wood. Between the secondary tissues, wood had five to seven times higher terpene production, than did liber (Baradat and Marpeau-Bezard, 1988).

Maritime Pine Terpene Emission

Foliage Emission. Only monoterpenes were detected in the needle emissions. α-Pinene, β-pinene, δ-3-carene, limonene, and myrcene, with low proportions of camphene and terpinolene were present (Table 1). Buchbauer et

TABLE 2. TERPENES IN MARITIME PINE WOOD, NEEDLE, AND LIBER RESINS AND IN NEEDLE AND PRUNING WOUND EMISSIONS

	Quantity (mean ± SE)				
Terpenes	Wood resin (μg/g resin)	Needle resin (μg/g needles)	Liber resin (μg/g liber)	Needle emission (μg/g needle/day)	Pruning wound emission (μg/cm^2 wound/day)
α-Pinene	92998.2 ± 8993.9	2325.8 ± 505.6	141.4 ± 29.9	4.4 ± 0.3	9.9 ± 1.2
Camphene	877.5 ± 79.2	25.4 ± 5.5	2.3 ± 0.4	0.1 ± 0.0	0.1 ± 0.0
β-Pinene	49338.6 ± 5272.8	4094.0 ± 881.1	76.0 ± 16.5	6.2 ± 0.6	5.3 ± 0.8
Myrcene	1820.5 ± 156.2	368.0 ± 79.1	3.3 ± 0.6	0.8 ± 0.1	0.3 ± 0.1
δ-3-Carene	38.9 ± 5.5	500.1 ± 118.7	0	1.6 ± 0.2	0.01 ± 0.0
Limonene	3149.0 ± 280.0	658.2 ± 142.8	6.1 ± 1.1	1.0 ± 0.1	0.3 ± 0.1
Terpinolene	521.2 ± 136.9	79.3 ± 25.6	3.9 ± 1.2	0.1 ± 0.0	0.01 ± 0.00
Linalool	0	0	0	0	0.03 ± 0.0
Longipinene	92.0 ± 28.8	0	0.1 ± 0.1	0	0
α-Copaene	20.0 ± 6.0	18.0 ± 3.5	0	0	0
α-Cubebene	163.5 ± 17.7	85.7 ± 14.9	0.1 ± 0.1	0	0
Longifolene	1046.4 ± 355.5	0	4.8 ± 2.0	0	0.01 ± 0.0
β-Caryophyllene	5299.9 ± 631.5	1383.4 ± 231.8	21.2 ± 5.5	0	0.01 ± 0.0
α-Humulene	892.1 ± 89.0	208.9 ± 32.5	5.8 ± 1.7	0	0
Germacrene D	28.5 ± 2.5	3467.0 ± 525.4	2.3 ± 0.4	0	0

al. (1994) also identified only monoterpenes in Douglas needle headspace (in decreasing proportions: sabinene, β-pinene, terpinolene, α-pinene, δ-3-carene, terpinene, and camphene). *Larix decidua* Mill. foliage emitted α-pinene, camphene, sabinene, limonene and β-phellandrene (Rappaport et al., 1995). Monoterpene emissions from maritime pine foliage averaged 14.4 μg/g/day on a fresh weight basis (Table 2), which is approximately equivalent to 28 μg/g/day on a dry weight basis (Porté, personal communication). For species that emit mainly monoterpenes, the emission rates usually range between 2.4 and 240 μg/day per leaf tissue dry weight (Lamb et al., 1985). Monoterpene emissions from five *Pinus* species ranged from 60 to 260 μg/g/day on a dry weight basis (Tingey and Burns, 1980).

In maritime pine, significant correlations were found between the same monoterpene proportions from needle emission and needle resin (Table 3), except for limonene. The greatest difference between the two profiles was the absence of sesquiterpenes from needle emissions. This absence could be explained by the fact that only a few monoterpenes, particularly α- and β-pinene, dominate terpene emissions during emission measurements (Zimmerman, 1979; Tingey et al., 1980; Evans et al., 1985; Juuti et al., 1990). The relative abundance of some monoterpenes in the gas phase has been attributed to their low boiling points (Hanover, 1972). When there is no difference in the diffusion coefficient between the two compounds, water solubility could also explain some of the variations in emissions rates found for the monoterpenes (Tucker and Nelken, 1982).

Pruning Wound Emission. Like needle emissions, maritime pine pruning wound emissions contained essentially α- and β-pinene and low proportions

TABLE 3. MATRIX OF CORRELATIONS OF PROPORTION OF SAME TERPENE IN TWO DIFFERENT PROFILES[a]

	Terpene								
Profile	API	CAM	BPI	MYR	D3C	LIM	TPL	LGF	BCA
Needle resin and needle emission	0.66 ***[b]	0.62 **	0.52 **	0.95 ***	0.83 ***	0.34	0.47 *		
Wood resin and pruning wound emission	0.55 **	0.25	0.68 ***	−0.42 *	0.34	−0.33	0.20	−0.14	0.32

[a]API: α-pinene, CAM: camphene, BPI: β-pinene, MYR: myrcene, D3C: δ-carene, LIM: limonene, TPL: terpinolene, LGF: longifolene, BCA: β-caryophyllene.
[b]Two-tailed significance: *0.05, **0.01, ***0.001.

of camphene, myrcene, limonene, and linalool. Traces of δ-3-carene, terpinolene and two sesquiterpenes (longifolene and β-caryophyllene) were also identified (Table 1). Riba (1991) has reported that the atmosphere above a maritime pine canopy contains α-pinene, β-pinene, δ-3-carene and limonene. Undamaged boles of *Pinus contorta murrayana* Engelm. emit β-phellandrene, ρ-cymene, β-pinene, an oxygenated monoterpene, and α-pinene (Rhoades, 1990). Terpene emissions from maritime pine pruning wounds averaged 16 $\mu g/cm^2/day$ (Table 2). Rhoades (1990) estimated the total volatile flux emitted by the boles of intact lodgepole pine at 6 $ng/cm^2/day$. It appears that pruning significantly increases terpene emissions. This increase could result from the elimination of bark as a natural barrier. Emissions could also have been enhanced by the neosynthesis of a traumatic resin (Cheniclet, 1987; Baradat and Marpeau-Bezard, 1988). In pruning wounds, the wood resin exuding from several resin ducts might mix with the resin of the induced reaction, although it has been demonstrated that terpene composition of the neosynthesized resin is very similar to that of wood resin (Cheniclet, 1987).

When terpene composition from pruning wound emissions and wood resin are compared, only a few correlations were found: for α-pinene, β-pinene, and myrcene proportions (Table 3). We also observed the almost complete absence of sesquiterpenes from wound emissions, which could be explained by the lower volatility and water solubility of these compounds compared to monoterpenes. However, the most important difference consisted in the presence of linalool, an oxygenated monoterpene, in the wound emissions, although this compound is not found in maritime pine resin. The presence of linalool might be an artifact. Pauly (1973) analyzed the volatile leaf oil of maritime pine and showed that some alcohols, especially linalool, seemed to be formed during the steam distillation process. In the method used, trapping was performed in situ for 48 hr in a region (southwestern France) where humidity is very high at night, even in summer. Terpenes are highly sensitive to oxidation and acid rearrangements on the adsorbent (Strömvall and Petersson, 1992). Terpenic compounds may also be transformed into oxygenated varieties through ozonolysis and interaction with °O and °OH radicals (Lilian, 1972; Graedel, 1979). In our trap, oxydants such as O_3, NO_3, etc., might have not been filtered. Added to the long air residence times in the enclosure, it is possible that they may have reacted on the trap. The proportion of wood resin terpinolene, which is often absent from surrounding air due to its high gas-phase reactivity (Strömvall and Petersson, 1992), was the only one that was both positively and significantly correlated with the proportion of linalool from the wound emissions ($r = 0.58$; $P = 0.003$). Moreover, measurements of needle emissions did not show a disappearance of terpinolene or an appearance of linalool under controlled conditions. A chemical transformation from terpinolene to linalool may occur in the trap.

Host-tree Susceptibility to Dioryctria sylvestrella

The terpene composition of the resins and pine emissions showed no significant quantitative or qualitative difference between *D. sylvestrella* trees that were attacked and not attacked. However, some differences in terpene proportions (semiquantitative) were significant (Figure 2). Attacked trees exhibited significantly more terpinolene in the wood resin ($df = 1$; Prob > $Z = 0.004$). Needle resin in attacked pines contained a significantly higher proportion of β-caryophyllene ($df = 1$; Prob > $Z = 0.001$) and a lower proportion of β-pinene ($df = 1$; Prob > $Z = 0.040$). Linalool ($df = 1$; Prob > $Z = 0.017$) and camphene ($df = 1$; Prob > $Z = 0.035$) were higher in the wound emissions of attacked trees while myrcene ($df = 1$; Prob > $Z = 0.026$) and β-caryophyllene ($df = 1$; Prob > $Z = 0.003$) were found in lower proportions. The terpene profile of the liber resin and needle emissions did not differ between attacked and unattacked maritime pines.

Because chemical control is not a realistic option (the pest is hidden under the bark almost all year) and biological control is uncertain, selection of resistant maritime pines represents a promising alternative method for pest management. This study pointed out seven candidates that could be used as biochemical markers in indirect selection programs: terpinolene, myrcene, camphene, linalool, β-pinene, and β-caryophyllene (twice). Because of the cumbersome sampling method, using terpene proportions from pruning wound emissions as biochemical markers is not feasible. Needle resin composition varies with age and season (Baradat and Marpeau-Bezard, 1988), and measurements would involve using the terpene proportion from needle resin as a biochemical marker. Among the seven candidates, terpinolene proportion in the wood resin is the easiest to measure and is genetically controlled (Kleinhentz et al., 1998). Its proportion is also stable at 7 or 8 years of age (Baradat et al., 1972).

Another promising method of pest management is to identify attractants that could be used in mass trapping. Short-range orientation towards and arrestment on host plants by insects may involve responses to volatile compounds emitted by the plant (Metcalf, 1987). Therefore, myrcene, β-caryophyllene, and β-pinene, which are absent or in higher proportions in the pine emissions from unattacked trees, cannot act as attractants. On the other hand, camphene, terpinolene, and linalool were good candidates for attractants. Linalool and terpinolene were the tested terpenes that induced the best electrophysiological response (the most stimulating) from *D. sylvestrella* females by EAG, while camphene was the monoterpene that induced the worst response (not significantly different from those elicited by the blank) (Jactel et al., 1996b). Because of the major role played by pruning wounds in host selection (Jactel et al., 1994, 1996a), the almost total absence of terpinolene from pruning wound emissions might appear to be inconsistent with its role as an attractant. However, this concurs with the

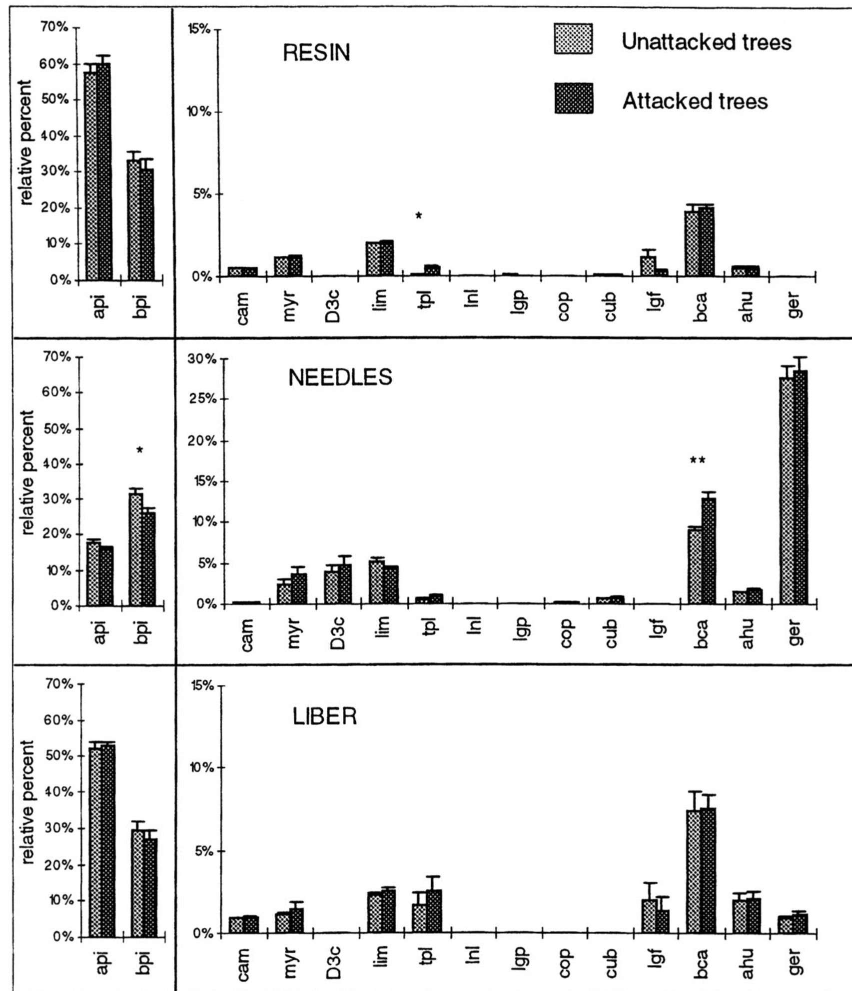

FIG. 2. Comparison of the mean terpene composition in wood resin, pentane extract (needles and liber), and headspace samples (needles and pruning wounds) from maritime pine trees attacked or unattacked by *D. sylvestrella*. Standard errors are indicated by vertical bars. One star above a pair of bars indicates a significant difference ($P > 0.05$). api: α-pinene, bpi: β-pinene, cam: camphene, myr: myrcene, D3c: δ-3-carene; lim: limonene, tpl: terpinolene, lnl: linalool, lgp: longipinene, cop: copaene, cub: cubebene, lgf: longifolene, bca: β-caryophyllene, ahu: α-humulene and ger: germacrene D.

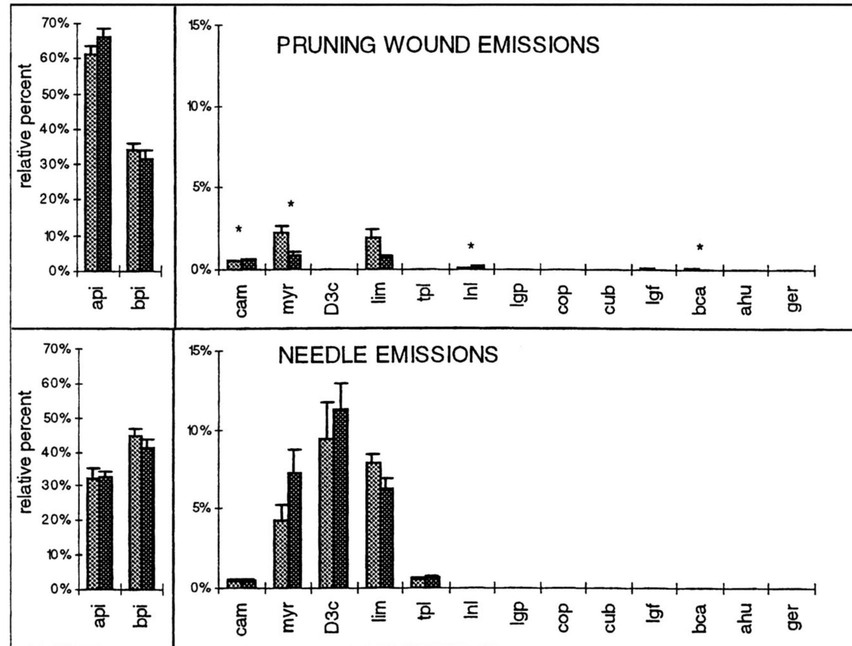

FIG. 2. Continued.

hypothesis of the high gas-phase reactivity of terpinolene and may be an artifact caused by technical problems. These compounds cannot be isolated as potential attractants. Moreover, terpinolene was the only terpene that was found in higher proportions and quantities in attacked trees than in all of the analyzed profiles (Figure 2). Kleinhentz et al. (1998) have also demonstrated the existence of a threshold for both tree diameter and terpinolene proportion in the wood resin under which trees are not subject to *D. sylvestrella* attack. Wright et al. (1975) have related the resistance of southern Scots pine varieties to *D. zimmermani* (Grote) to the low levels of δ-3-carene and terpinolene, and many studies (Fatzinger and Merkel, 1985; Hanula et al., 1985; Shu et al., 1997; Sadof and Grant, 1997) support the hypothesis that monoterpenes contribute to attacks by *Dioryctria* species. Further behavioral experiments should be developed to verify the real attractiveness of camphene, terpinolene, and linalool in the maritime pine–*D. sylvestrella* relationship and evaluate their potential use in pest management.

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MINOR AND INTERMEDIATE COMPONENTS ENHANCE ATTRACTION OF FEMALE MEDITERRANEAN FRUIT FLIES TO NATURAL MALE ODOR PHEROMONE AND ITS SYNTHETIC MAJOR COMPONENTS

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Abstract—The attraction of virgin female medflies to either the natural pheromonal odor of calling males or its synthetic major components was enhanced by both intermediate and minor pheromonal components in multiple choice discrimination tests. The modification of the standard Gow rotating-trap-array, cage olfactometer to allow a single source of natural pheromonal odor to be delivered equally and simultaneously to a number of traps greatly increased trapping efficacy (64% capture rate) and ability to resolve odor preference discrimination by female flies. In olfactometer cage bioassays, responding female medflies expressed preferences in attraction to male odor augmented with either synthetic intermediate or minor components over male odor alone. In dual-choice flight-tunnel bioassays, the minor blend enhanced the attractiveness of both the natural male odor and its synthetic major components. Moreover, the minor blend, when presented together with the synthetic major components, comprised an artificial pheromonal lure competitive for the first time with the natural male odor in attraction of virgin female medflies.

Key Words—Diptera, Tephritidae, Mediterranean fruit fly, *Ceratitis capitata*, pheromone, male odor, attractant, minor components, multiple choice olfactometer, flight tunnel.

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INTRODUCTION

The Mediterranean fruit fly (medfly), *Ceratitis capitata* (Wiedemann), is a threat to subtropical regions of the world, including part of the continental United States. Both the detection of tephritid invasions and eradication of infestations utilize olfactory attractants. Key detection tools and control strategies presently needed for medflies are lures specifically attractive to females (Light, 1995). Food-based synthetic attractants have recently drawn interest as improved medfly monitoring lures (see references in Epsky et al., 1999). Although these food-based synthetic lures are biased toward female attraction, they are not specific for it, with the percentage of female medflies attracted being ca. 75% (a slight improvement over the 72% female capture rate of traditional hydrolyzed protein baits) (Epsky et al., 1999). Female-specific attractants would augment current male lures and food-based lures, help detect beginning invasions, and help resolve questions about female reproductive status of a population. In particular, sex pheromone-based attractants for virgin mate-seeking females and host-fruit-based attractants for gravid ovipositional females would allow differentiation of populations of reproductively immature versus mature females (Jang, 1995; Light, 1995; Jang and Light, 1996b; Light and Jang, 1996). A key medfly eradication strategy is the sterile insect technique (SIT), wherein mass-reared males are released to call, attract, and mate with the targeted feral females. The success of SIT is predicated on the attractiveness of the male-emitted sex pheromone. Thus, there is a crucial need to resolve the chemical composition and attractiveness of the medfly male pheromone and to develop and optimize its use as a detection and control tool.

Courtship behavior for medflies was initially described by Martelli (1910), while Back and Pemberton (1918) first suggested that the pungent "stale mucus-like" odor of male medflies might be a sex attractant. Féron (1959, 1962) established that the male odor is a true sex pheromone (i.e., attractive to females and critical in courtship and mate acceptance). Since these behavioral observations, several identifications of volatile components from calling male medflies have been reported (Jacobson et al., 1973; Baker et al., 1985; Jang et al., 1989; Heath et al., 1991; Flath et al., 1993). The compounds identified by Jacobson et al. (1973) are not considered in this report because they attract primarily males (Ohinata et al., 1977) and were not detected in subsequent chemical analyses (Baker et al., 1985; Jang et al., 1989; Heath et al., 1991; Flath et al., 1993). Baker et al. (1985) identified nine components in the medfly male odor [four major: 1-pyrroline, (*E*,*E*)-α-farnesene, ethyl (*E*)-3-octenoate, and geranyl acetate; an interme diate: (*E*)-2-hexenoic acid; and four minor: dihydro-3-methylfuran-2(3*H*)-one, 2-ethyl-3,5-dimethylpyrazine, linalool, and ethyl acetate]. No bioactivity data were given, but Baker et al. (1985) stated that although mixtures of components were attractive to females, only 1-pyrroline elicited attraction when presented

singly. The identification of these nine male odor components was confirmed by Jang et al. (1989) and Flath et al. (1993), who expanded the compositional complexity of the odor to 66 identified components (5 major, 6 intermediate, 12 minor, and 43 trace components).

The natural pheromonal odor emitted by confined males (e.g., 5–25 males) is well established as an adequate long-range attractant for virgin females in both field (Ohinata et al., 1977; Wong and Nakahara, 1978; Zümreoglu et al. 1979; Nakagawa et al., 1981) and laboratory tests. Male odor elicits zigzagging upwind flight orientation by virgin females in flight-tunnel bioassays (Landolt et al., 1992; Jang et al., 1994; Jang, 1995). However, various synthetic components of male odor were found to be weak attractants, eliciting variable and reduced degrees of female attraction relative to: (1) the natural male odor in laboratory bioassays (Landolt et al., 1992; Jang et al., 1994), (2) field capture of males with trimedlure (Baker et al., 1990; Heath et al., 1991; Howse and Knapp, 1995), or (3) field capture of both sexes with ammonia (Baker et al., 1990).

Male odor was a much more effective attractant in flight tunnel bioassays than any single major component (Jang et al., 1994) or ensembles of three to five synthetic compounds identified as major components of the male odor (Landolt et al., 1992; Heath and Epsky, 1993; Jang et al., 1994). In no-choice flight-tunnel tests, Landolt et al. (1992) showed that male odor was far superior to synthetic lure with three major components (Heath et al., 1991) in eliciting flight orientation from mature virgin female medflies (2.5 × more flights/fly, 3.4 × greater zigzagging upwind flight, and 13.1 × greater contact with odor source). In dual-choice flight-tunnel bioassays that competitively tested attraction and female preference, male odor-emitting traps captured 2.9 × more virgin females than traps emitting an ensemble of the five major components (Jang et al., 1994).

To date, all synthetic lures based on identified major components of male odor have limited attractiveness for virgin female medflies. Therefore, there is a need to increase the efficacy (while maintaining species and sexual specificities) of a sex pheromone-based female medfly attractant if it is to be developed into an effective detection lure. In addition, if more competitive and competent sterile males are to be selected for SIT, then the active pheromonal attractants in the male odor need to be defined. We report here in detail our initial investigations (Jang and Light, 1996a) into whether additional components of the male odor, i.e., intermediate or minor components, can enhance pheromonal attraction of virgin female medflies.

The objectives of this study were to determine: (1) if intermediate and minor components identified in the male odor will enhance the attractiveness of either the natural male odor or its synthetic major components (Jang et al., 1994), (2) if this enhancement is dose dependent, and (3) which male odorants might be responsible for enhancing the attractiveness of the natural pheromone. We carried out these objectives through a unique experimental paradigm where female

attraction and preferences were tested by competitive multiple choice treatments of male odor alone or synthetic major components alone versus these same standards augmented with intermediate and minor components.

METHODS AND MATERIALS

Insects. Pupae of *C. capitata* were supplied from the colony at the mass-rearing facility, USDA, ARS Tropical Fruit and Vegetable Laboratory, Honolulu, Hawaii. The pupae were sexed (Cunningham, 1966) and sexes kept segregated as both pupae and emergent adult flies. Groups of 50 pupae were placed in plastic cups (11.5 cm diam. × 7.5 cm deep) with nylon mesh covers. Adults were provided sucrose, hydrolyzed protein, and water throughout their development. Rooms (ca. 20–25°C, 60–70% relative humidity, and 06:30–18:30 photophase) in three buildings were used to separate males from females and both from the bioassay facility. The segregation of the sexes guaranteed that the female flies were unmated and had experienced no exposure to male-produced sex pheromone. Cohorts of flies, from shipments of pupae, emerged after one to two days. They were allowed to reach sexual maturity [ca. five days after emergence for lab reared flies (Wong and Nakahara, 1978)] without mating and then were tested as a designated postemergence age class. These age classes of unmated *C. capitata* ranged from 5–6 days to 9–10 days after emergence (70% were 7–8 days or 8–9 days).

Test Volatiles. The synthetic test volatiles were constituents of medfly male odor (Baker et al., 1985; Jang et al., 1989; Flath et al., 1993). Experiments on the inherent attractiveness of the designated major constituents of male odor (accounting for a combined 93.6% of total quantity of odorants trapped) (Jang et al., 1989; Flath et al., 1993) were reported elsewhere (Jang et al., 1994). The primary focus of this research was to evaluate the synthetic volatiles designated by Jang et al. (1989) as either intermediate constituents (combined 4.8% of identified odorants, individually from 0.6% to 1.3%) or minor constituents (combined 1.6% of identified odorants, individually from <0.1% to 0.5%) of the male odor (Flath et al., 1993). Because of the difficulty in formulating all constituents individually (five intermediate and nine minor constituents) and testing their interactive effects, we formulated and tested six distinct, liquid blends (Table 1): (1) two category blends, either a blend of five intermediate constituents (intermediate blend), and a blend of nine minor constituents (minor blend), and (2) four subcategory blends, based on a common chemical structure or functional moiety (i.e., octenoates, monoterpenoids, and their esters).

Table 1 lists the commercial or synthetic sources and purities of the intermediate and minor constituent test chemicals. The method of Linstead et al. (1933) was used to synthesize (*E*)-3-hexenoic acid and (*E*)-3-octenoic acid. These were

TABLE 1. INTERMEDIATE AND MINOR VOLATILE CONSTITUENTS IDENTIFIED IN THE PHEROMONAL ODOR OF CALLING MALE MEDITERRANEAN FRUIT FLIES; THE SYNTHETIC CONSTITUENTS' SOURCE, PURITY, RETENTION TIME-VOLATILITY INDEX, AND ELECTROANTENNOGRAM RESPONSIVENESS OF FEMALES; AND PERCENT COMPOSITION OF THE BLEND RECIPES AND HEADSPACE OF THE SYNTHETIC INTERMEDIATE AND MINOR CATEGORY AND SUBCATEGORY BLENDS

			Percent Composition of Category Blend Constituents in			Percent Compositional Recipes for Subcategory Blends (Vol:Vol)			
Constituents:	Source[a] Purity-Retention Time[b]	EAG Potency ($\bar{x}$% of Response to Standard[c])	Headspace of Natural Medfly Male Odor[d]	Recipes of Synthetic Category Blends (Vol:Vol)[e]	Headspace of Synthetic Category Blends[f]	"Intermediate Monoterpenoid Blend"	"Minor Octenoates"	"Minor-Mono-terpenoid Esters"	"Minor Other Compounds"
Intermediate category:									
Myrcene	(A)-96.5%-1605	168%	16.0%	8.5%	12.7%	10.2%			
Ethyl(E)-3-Hexanoate	(B)-97.8%-1632	268%	4.1%	6.7%	6.7%				
(Z)-β-Ocimene	(C)-94.0%-1751	(Not tested)	35.4%	21.9%	28.1%	20.7%			
(E)-β-Ocimene	(A)-81.7%-1783	131%	20.3%	21.5%	26.8%	20.7%			
Linalool	(A)-97.0%-1922	112%	24.2%	41.4%	25.7%	48.4%			
Minor category:									
Propyl Acetate	(D)-98.0%-666	68%	10.3%	0.7%	2.8%				7.1%
Pyrrole	(A)-99.0%-786	64%	24.4%	0.9%	4.3%				7.1%
3-Methylbut-3-enyl Acetate	(B)-88.0%-1223	(Not tested)	11.0%	0.6%	2.0%				14.3%
6-Methylhept-5-en 2-one	(E)-99.9%-1542	172%	5.7%	3.1%	11.7%				71.4%
Isopropyl(E)-3-Octenoate	(B)-97.6%-2317	142%	5.7%	14.8%	20.3%		30.5%		
Ethyl(E)-2-Octenoate	(B)-96.6%-2337	158%	9.0%	15.4%	19.0%		27.1%		
Linalyl Acetate	(A)-97.0%-2379	99%	6.2%	17.7%	4.0%			33.3%	
Propyl(E)-3-Octenoate	(B)-96.1%-2472	135%	9.5%	21.7%	18.0%		42.4%		
Methyl Geranate	(F)-95.3%-2545	75%	18.2%	25.1%	17.9%			66.7%	

[a]Sources of compounds: A, Aldrich Chemical Co.; B, synthesis by R. G. Binder; C, isolated by R. A. Flath; D, Chemical Supply Co.; E, Synthesis by R. A. Flath; F, purified from Bedoukian.

[b]Capillary GLC analysis (12.5m × 0.2mm methyl silicone cross-linked column, DB-1), with retention time an index of relative volatility between constituents.

[c]Data from Jang *et al.* (1989), mean percent EAG response of female medfly antennae to 100μg doses relative to a 10μg dose of a hexan-1-ol standard.

[d]Estimated proportions of category constituents based on GLC integration of the headspace Tenax trapping of the natural pheromonal odor of calling male medflies; Jang *et al.*, 1989, Flath *et al.*, unpublished.

[e]Volume to volume percentage-based recipes for blending neat, liquid proportions of the constituents to create synthetic blends.

[f]The relative percent composition of the headspace, Tenax-trapped, odor that evaporated from 1μl capillary formulations of the synthetic blends.

esterified with ethanol, propanol, or isopropanol and purified by distillation. (*E*)-2-octenoic acid (Aldrich Chemical Co.) was esterified with ethanol, and the ester was purified by chromatography on silica gel. Acetyl chloride in pyridine reacted with 3-methylbut-3-enol to provide 3-methylbut-3-enyl acetate.

The percentage-based liquid (volume–volume) recipes used for formulating the intermediate and minor constituent blends are defined in Table 1. The recipes for the two blends of intermediate components mimicked the analyzed proportional composition of these constituents in the headspace-trapped natural male odor (Jang et al., 1989; Flath et al., 1993). The liquid volume ratios of each blend's constituents were adjusted empirically based on the relative vapor pressures of these volatiles and on headspace (Tenax) trappings and GC analyses of preliminary blends, followed by reformulation and repeat analysis. The goal and procedures for formulating the three "subcategory blends" of minor constituents was similar to the above, but the formulation of the nine-component minor blend was intentionally biased in its proportions to create a synthetic headspace odor that allowed the less volatile constituents to exceed the contribution of the most volatile constituents (Table 1). The chief rationale was that these six, less volatile, larger molecules had elicited far greater electroantennogram responses (>2.5×) than the three most volatile constituents (Jang et al., 1989) (Table 1).

The intermediate and minor blends were loaded into various sizes and configurations of tubes and capillaries to achieve distinct, stable, and measurable rates of evaporation (Table 2). The large glass tube (3.30 mm ID × 1.0 cm long) was open at the top and flame-sealed at the bottom. Open-ended glass capillaries (Microcaps, Drummond Scientific, Inc.) were 3.25 cm long and contained either 1 μl (0.19 mm ID) or 5 μl (0.44 mm ID) when completely filled. Evaporation rates in the capillaries were measured after each test replication by measuring the millimeter drop in the meniscus. Mean rates of evaporation for the formulations were calculated from an average density (micrograms per microliter) for each blend derived from the density and proportions of each constituent (Table 2). Different levels of evaporation were achieved by using various formulations that varied in: (1) tube/capillary ID size, (2) fill level of liquid versus empty headspace, and (3) evaporative openings (top and bottom versus top alone) (Table 2). These formulations were in partially filled sealed bottom large tubes, completely filled open ended 5-μl and 1-μl capillaries, and partially filled (ca. 1-cm empty headspace above the initial meniscus) sealed bottom (ca. 3-mm plug of plasticine clay) 5-μl and 1-μl capillaries.

The five major components were not dissolved together in a single blend but each compound was allowed to evaporate individually from its own tube or capillary, bundled together to emit an ensemble odor (Jang et al., 1994). The five major components (their formulations and average emission rates) were 1-pyrroline (tube, 168 ng/hr), ethyl acetate (1-μl plugged capillary, 11.0 μg/hr),

TABLE 2. MEAN EVAPORATION RATES[a] (μg/hr) OF NEAT INTERMEDIATE AND MINOR CATEGORY BLENDS, SUBCATEGORY BLENDS, AND INDIVIDUAL COMPOUNDS EMITTED FROM THE VARIOUS TUBE AND CAPILLARY SOURCES

Blend or Compound	Tube[b] $\bar{x}$ ± SEM	5 μl Capillary[c] $\bar{x}$ ± SEM	1 μl Capillary[c] $\bar{x}$ ± SEM	5 μl Plugged Capillary[d] $\bar{x}$ ± SEM	1 μl Plugged Capillary[d] $\bar{x}$ ± SEM
Intermediate Blend	3,344 ± 774	307 ± 44	45 ± 3	18 ± 3	5 ± 1
Intermediate, Monoterpene Blend			34 ± 6		
Ethyl (E)-3-Hexenoate			93 ± 12		
Linalool			17 ± 4		
Minor Blend	3,295 ± 874	168 ± 23	17 ± 1	12 ± 3	3 ± 1
Minor, Octenoate Blend			12 ± 2		
Minor, Monoterpenoid-Esters Blend			7 ± 1		
Minor, Other-Compounds Blend			47 ± 3		
6-Methyl hept-5-en-2-one			43 ± 11		

[a]Mean evaporation rates based on 6 to 45 replicate measurements, of the linear drop in meniscus from initial fill line and/or tube weight loss.
[b]Tube: open top, sealed bottom tubes (3.30 mm I.D.) partially filled to leave a 5 mm headspace (or empty tube space from rim to initial meniscus).
[c]5 μl and 1 μl Capillaries (0.44 and 0.19 mm I.D.): open at both ends, vertically positioned and initially completely filled.
[d]5 μl and 1 μl Plugged Capillaries (0.44 and 0.19 mm I.D.): vertically positioned, capillary tubes with bottom-end plugged with plasticine clay and capillaries only partially filled, creating an initial 10 mm headspace below the top opening.

ethyl (*E*)-3-octenoate (5-μl capillary, 5.14 μg/hr), geranyl acetate (1-μl plugged capillary, 1.47 μg/hr), and (*E,E*)-α-farnesene (two 1-μl capillaries, 1.98 μg/hr). All major components were >97% pure, with ethyl acetate and geranyl acetate obtained from Aldrich Chemical Co., 1-pyrroline and ethyl (*E*)-3-octenoate synthesized (RGB), and (*E,E*)-α-farnesene isolated from ylang-ylang oil (RAF) (Jang et al., 1994).

Multiple-Choice Cage Olfactometer Bioassays. Multiple-choice cage olfactometers were used to assess whether the atractiveness of the naturally emitted male odor could be augmented with synthetic blends of its constituent volatile compounds. Female flies were released into a cage arena and allowed to choose between landing on traps emitting male odor alone or on traps emanating male

odor plus test blends of synthetic volatiles. Response preferences to qualitative and quantitative aspects of the supplemental volatiles were assessed.

The multiple-choice olfactometers were a modification of the Gow arena (Gothilf and Galun, 1982; Gow, 1954; Jang and Nishijima, 1990), comprised of a cage housing a horizontally rotating, spoked hub on which traps were suspended. The two olfactometer cages had access doors and aluminum window screening on all sides and were either 0.45 m^3 (77 × 79 × 74 cm) or 0.78 m^3 (92 cm/side) in volume. A variable-speed, DC motor with a reduction gear (G. K. Heller Co.) was mounted above the center of each cage, and rotated an aluminum hub (25-cm pie pan) from which six to eight rods or spokes radiated outward (20 cm). The hub was 7 cm below the cage ceiling. Traps were suspended from the spokes, and the hub system rotated at 0.5 rpm.

Our chief modification to the Gow olfactometer was to permit a single-source naturally emitted odor to be divided equally and simultaneously to a number of the olfactometer traps. An airstream carried the odor into the olfactometer cage and delivered it to the rotating hub system, where it emanated from all or a number of the rotating traps.

For each test session, a cup (with a cloth mesh lid) containing 50 sexually mature male flies of a set age-class was placed in a sealed 10.5-liter glass desiccator chamber with inlet and outlet airflow ports. Males were placed in the chambers between 06:45 and 07:45 hr, and a series of experiments was conducted between ca. 08:00 and 12:30 hr. Male flies readily called (50–90% at any instance) and released pheromone, as evidenced by eversion of their anal ampoule and rapid and pulsed forward wing fanning (Lhoste and Roche, 1960; Féron, 1959, 1962).

Compressed air from a pressurized tank was filtered through charcoal and regulated to flow through the chamber at rates of ca. 400–800 ml/min. The entrained male odor was split into two equal airstreams and delivered via Teflon tubing (7 mm OD) to two glass transference flasks, each located directly below the central axis of an olfactometer cage. Within each olfactometer cage, a stainless-steel rigid tube (7 mm OD, 80 or 100 cm long) was inserted into and extended up from the transference flask through the cage floor to the underside center point of the rotating hub, where it was secured to the end of the motor shaft. Each transference flask received an inflow of the male odor from the male calling chamber and allowed the odors to flow up the rotating rigid tube to the spoke array. The glass transference flask (test tube shaped, 11 cm × 3 cm ID) had a glass tubing (6 mm OD) inlet side port and a large outlet top port. The top port was a ground-glass fitting accommodating a Teflon cork (inlet adaptor; Kontes, Inc.), with a central bore (ca. 8 mm ID) fitted with a screw cap and O-ring, that provided a pressure air seal for the rotating rigid tube.

On the underside of the hub array within the cage, the male odor entered a splitter system of T-fittings (Swagelok, Crawford Fitting Co., Solon, Ohio) that

divided the flow equally to four, six, or eight 3.5-mm-OD Teflon tubes, each of which terminated in a suspended trap, where the air carrying the male odor flowed out. All tubing and the fittings of each arm of the splitter system were identical in diameter and length to provide equal flow rates to each test trap in an experimental array. Before each experiment, precision flowmeters (Cole Parmer Instrument Co.) were used to monitor the outflow of each trap tube to ensure equality of flow rates (ca. 50 ml/min/trap tube).

Small delta traps (ca. 6.4 cm long), with sticky-glue (Stikem Special, Seabright, Inc.) floor inserts, were fabricated by cutting in half the standard monitoring trap (Jackson trap; Sandia Die & Cartridge Co.). The sticky inserts were replaced after each test, and the entire trap was replaced after each morning's replicated experiment.

The trap stimuli used in the multiple choice cage olfactometer tests were: (1) blank traps (without male odor and/or test blends/compounds); (2) male odor emitting traps; (3) male odor emitting traps that coemitted the odor of either a category blend, subcategory blend, or individual test compound; and (4) traps emitting the odor of a blend or test compound without the emission of male odor.

Each test replication commenced with the release of either 50 or 100 virgin female medflies into an olfactometer cage. The duration of each test was 30 min. After the test period, the nontrapped female flies were counted and removed (via vacuum cleaner) from the cage, the trapped flies were counted and the sticky trap inserts were removed and replaced, the drop in meniscus was measured within the treatment capillaries, the treatments were moved to new, random positions on the hub spoke array, the flow rates from the trap tubes were measured to ensure equality, and the percentage of males calling in the confinement chamber was estimated.

Each experiment was replicated 5–10 times. Treatment captures were expressed as a mean percentage of total flies released. For statistical testing the mean percent capture data were log transformed and then compared through ANOVA followed by Duncan's multiple range test and Student-Newman-Keuls test of ranked data ($P \leq 0.05$) (SAS Institute, 1988).

Dual-Choice Flight-Tunnel Bioassays. A laminar flow (0.15 m/sec) rectangular ($0.9 \times 0.9 \times 2.8$ m), tempered glass flight tunnel was used (Jang and Light, 1991; Jang et al., 1994). Dual-choice, discrimination, and preference bioassays were conducted to directly and competitively test the ability of the minor blend to augment and enhance the attractiveness of either the natural medfly male odor or the odor of its synthetic five major components.

Calling males (40 or 50) or the synthetic major components were placed in glass desiccator chambers outside the flight tunnel. The effluent odors were delivered to and emitted from one or both of two artificial leaf model traps hung in the upwind end of the flight tunnel (Jang et al., 1994). Traps were sticky glue-

coated, green paper leaf models, comprised of two leaves (each 15 × 23 cm) attached to a central odor emission chamber (polyethylene bottle with bottom removed). For most experiments, the entrained odor, either male odor or synthetic major components, was split and delivered to the emission chambers of both leaf models. The minor blend was released from 1-μl capillary tubes (both ends open) taped to the inner wall of the emission chamber of the assigned leaf model. Four distinct dual-choice experiments were conducted: male odor versus male odor + minor blend, male odor versus synthetic major components, male odor versus synthetic major components + minor blend, and synthetic major components versus synthetic major components + minor blend.

For each test, 50 females were released, and after 30 min, captured flies were counted and nonresponders were vacuumed from the tunnel. Five to 13 replications were conducted per experiment. Numbers of females captured in the dual traps were compared by using a paired t test of means.

RESULTS

Multiple-Choice Cage Olfactometer Bioassays. The modification to deliver male odor to an array of rotating traps in a cage olfactometer greatly increased the trapping efficacy over previous experiments that were limited to testing only synthetic compounds. Within the 30-min test period, this olfactometer system trapped on average 64.3% (±1.9 SEM for 58 replicates) of the released female medflies when male odor was emitted from the traps versus a total fly capture of 13.3% (±1.7 SEM for 53 replicates) when only synthetic pheromonal components were tested without male odor (Jang et al., 1994). The efficacy of this improved bioassay arena is demonstrated by the rapid rate at which the virgin female flies responded and were captured in traps in the male odor augmentation tests (Figure 1). About 71% of the female flies captured in a 30-min test were captured within the first 10 min and ca. 90% within 20 min. Male and female fly age (within the narrow 5- to 10-day-old range tested) had no significant effect on percentage of females captured per test (ANOVA, $P = 0.54$), nor did the time of the day (ca. 8–12 AM) ($P = 0.27$).

In the multichoice cage arena (for these tests: six traps, three treatments, two replicate traps per treatment), more virgin female medflies were captured in the traps emitting male odor augmented with the intermediate blend of male odor components (29.6% ± 3.5 SEM of females released) than traps emitting male odor alone (17.2% ± 1.2) or no odor blank traps (4.8% ± 0.9) (Figure 2A). By using the same experimental design, more virgin female medflies were captured in the male odor traps augmented with the minor blend of male odor components (36.0% ± 3.5 of females released) than in either male odor alone (16.0% ± 2.6) or blank traps (7.3% ± 1.2) (Figure 2B). When presented with the

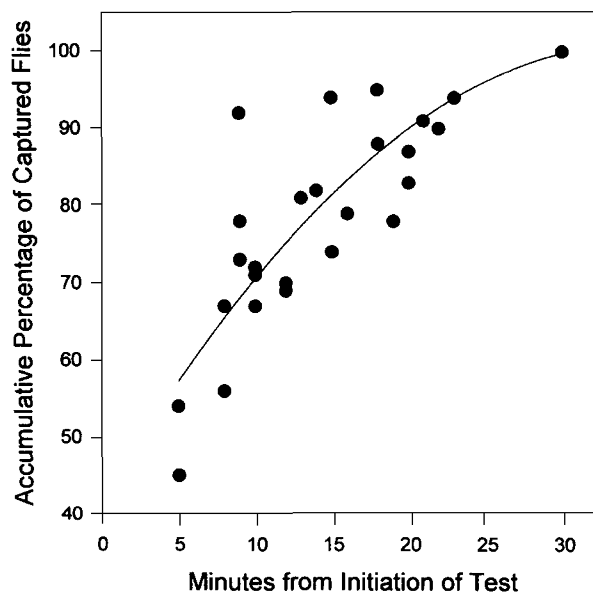

FIG. 1. Response dynamics or the rate at which virgin female medflies were captured in pheromone bioassays conducted in the multiple-choice cage olfactometer, modified to deliver male odor from the same entrainment source to a number of rotating traps simultaneously.

three-treatment choice of male odor alone traps or male odor traps augmented with the intermediate or minor blends, more virgin female flies were captured in either of the synthetic odor augmented male odor traps than in male odor alone traps (Figure 2C). The female capture in traps emitting male odor augmented with the minor blend did not significantly exceed the enhancement of male odor by the intermediate blend (26.5% vs. 22.5%).

The enhancement of male odor attractiveness was shown to be dose dependent in competitive multiple choice tests (Figure 3). The mean evaporation rates for the intermediate and minor blends from the capillary and tube formulations spanned three orders of magnitude, ranging from ca. 3 μg/hr to >3 mg/hr (Table 2). For both blends, the formulation that was most preferred by responding females and that elicited the greatest enhancement of male odor attractiveness was from 1-μl capillary tubes (fully filled open-ended) that generated evaporation rates of ca. 17 μg/hr and 45 μg/hr for the minor and intermediate blends, respectively (Figure 3).

Multiple-choice experiments of female preference to male odor + intermediate or minor category blends versus their various subcategory blends and certain

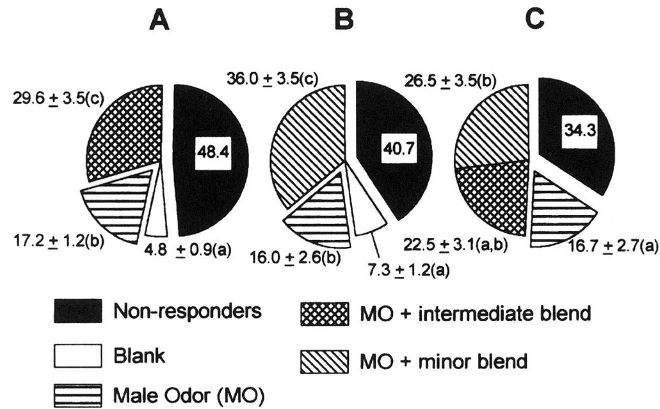

FIG. 2. Preference and attraction of virgin female medflies to male odor augmented with blends of identified intermediate and minor components versus male odor alone in a multiple-choice cage olfactometer. Percent capture (± SEM) of females to either male odor alone or male odor augmented with the intermediate blend (A), minor blend (B), and competitively either intermediate blend or minor blend (C). (Mean values followed by the same letter are not significantly different).

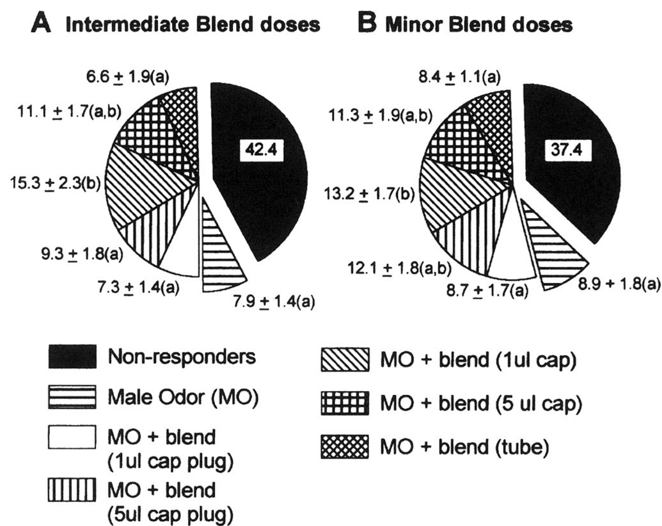

FIG. 3. Preference and attraction of virgin female medflies to male odor augmented with blends of identified intermediate and minor components evaporating at different release rates versus male odor alone in a multiple choice, cage olfactometer. Percent capture (± SEM) of females to either male odor alone or male odor augmented with five distinct evaporation rates of the intermediate blend (A) and minor blend (B).

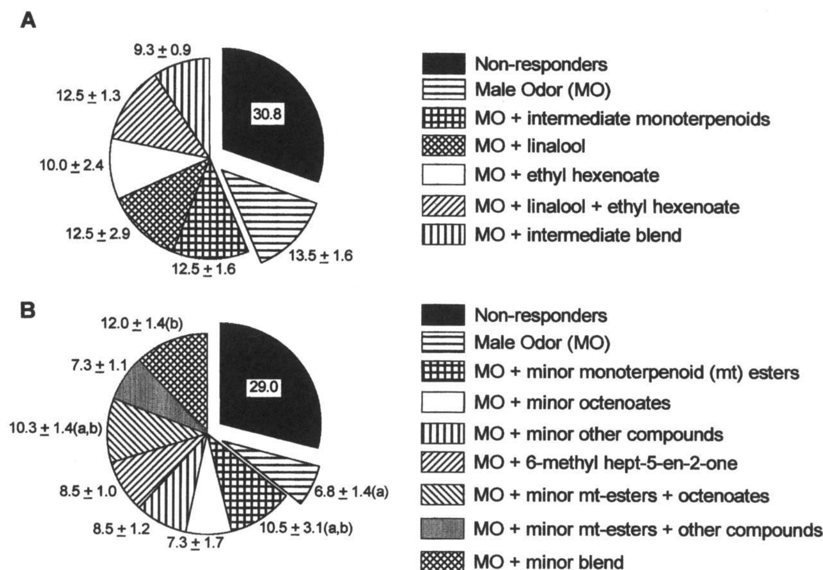

FIG. 4. Preference and attraction (percent capture ± SEM) of virgin female medflies to male odor alone versus male odor augmented with either the intermediate (A) or minor (B) category blends, subcategory blends, and individual components in a multiple choice, cage olfactometer (see Table 1 for blend composition).

individual constituent compounds did not resolve or identify which subcategory blends or compounds were responsible for enhancement of male odor attractiveness (Table 1; Figure 4). Only the minor blend formulation enhanced male odor attraction significantly over male odor alone or over the various minor constituent subcategory blends (Figure 4B).

Dual-Choice Flight-Tunnel Bioassays. More female flies were attracted to traps emitting male odor augmented with the minor blend (1-μl capillary) than traps emitting male odor alone (Figure 5). Female flies preferred (2.9 × more females attracted) male odor over the synthetic major components as an attractant (Figure 5). However, when the ensemble odor of the major components was augmented with the minor blend and competitively paired against male odor, female flies showed no significant difference in attraction between the synthetic complex pheromonal odor and the natural pheromone (Figure 5). The minor blend also enhanced the attractiveness of the major components. Thus, the additional odor of the minor blend enhanced the attractiveness of both the natural male odor and the synthetic major components to responding female medflies.

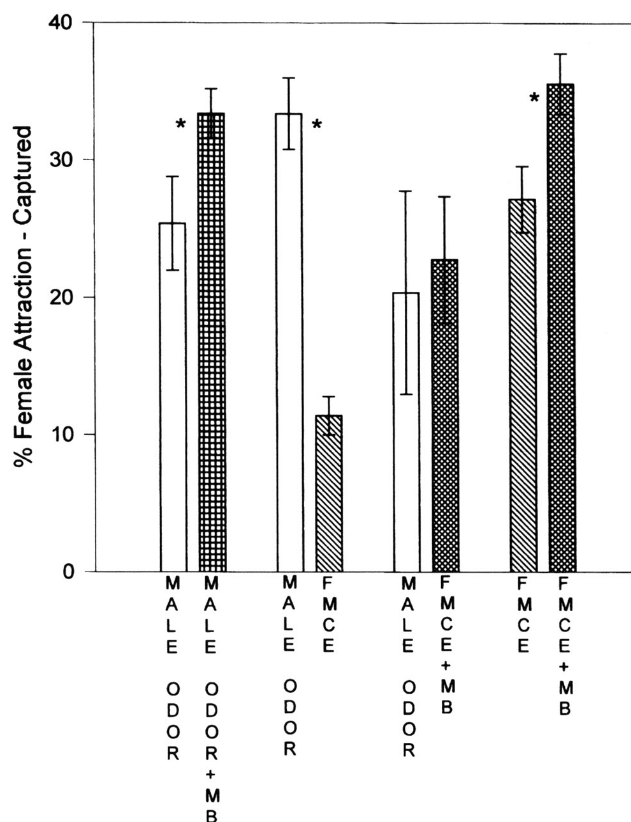

FIG. 5. Preference and attraction of virgin female medflies in a series of four dual-choice tests in a flight-tunnel arena, pairing either male odor or the synthetic five major component ensemble (FMCE) versus the same augmented with the minor blend (MB). *Significant difference between the paired treatments, $P < 0.05$.

DISCUSSION

Our modification of the Gow-designed rotating-hub, multiple-trap-array olfactometer (Gow, 1954; Gothilf and Galun, 1982) resulted in a bioassay system with a number of unique attributes, strengths, and limitations. This bioassay system: (1) tests competitively and simultaneously, in an array of traps, the same natural odor emitted from a single common source, (2) allows this natural odor to be selectively augmented with treatment odorants for odor discrimination and preference choice screening, (3) is capable of behavioral resolution of subtle to substantial odor changes/distortions, (4) is capable of relatively rapid and effi-

cient trapping of test insects and resolving behavioral responses and preferences, and (5) is prone to limitations of "discrimination overload," i.e., too great an exposure to either odorant concentrations and/or similar odorant choices. Our flight-tunnel bioassay system has similar attributes and strengths, while its limitations is the increased number of required experiments to test all the binary combinations of treatments and standards.

Both the intermediate and minor blends enhanced the attractiveness of male odor to mature virgin female medflies. The male odor + intermediate blend out-competed male odor alone, capturing 1.7× more females, and the male odor + minor blend out-competed the male odor alone, capturing 2.3× more females. However, in the three-choice direct competition tests, the minor blend equaled the intermediate blend in enhancing the attractiveness of the male odor. Considering that the quantitative delivery or evaporation rate of the minor blend was ca. 1/3 that of the intermediate blend (17 μg/hr vs. 45 μg/hr in the standard, 1-μl capillary formulations) the minor blend may also be potent. In this multiple choice paradigm, virgin female medflies preferentially responded and were apparently able to discriminate both the qualitative presence and the proportional or increased amounts of these supplemental intermediate and minor components over their inherent levels in the natural male odor.

The multiple-choice preference tests did not resolve or identify which of the subcategory blends or individual compounds were responsible for enhancement of male odor attractiveness by their parent category blends. This was possibly due to the greater number of treatment choices, potential sensory adaptation to pheromone, and discrimination overload of the experimental design with six or eight odor-emitting traps presented simultaneously in the multiple-trap arrays. The results support the conclusion for the minor blend tests that the greatest enhancement activity resides with the complete minor category blend and not with the tested minor subcategory blends and individual compounds. Thus, full enhancement of female attraction appears to depend on pheromone complexity, and the presence of multiple components and diverse chemical structures. Our preference test results suggest the possibility of male odor enhancement activity by the monoterpenoid esters (linalyl acetate and methyl geranate) that are minor constituents of the male odor (Jang et al., 1989; Flath et al., 1993). Male odor enhancement was not observed for linalool, a monoterpenoid alcohol in the intermediate subcategory blend preference tests. The occurrence of linalool in male odor, however, is well established (Baker et al., 1985; Jang et al., 1989; Flath et al., 1993; Crossé et al., 1995), and it is attractive both as a single compound for both sexes of medflies (Howse, 1987; Baker et al., 1990) and in a blend with the five major components for virgin females (Jang et al., 1989).

The dual-choice, flight-tunnel bioassays confirm that the minor blend enhances the attractiveness of both the natural male odor and the synthetic major component lure. Moreover, the minor blend, when evaporated with the major

components, increased attractiveness so that the combination was directly, and for the first time equally, competitive with the natural male odor in laboratory bioassays. In numerous prior laboratory tests, female capture efficacy of the major components alone was on average 2.9× less attractive than the male odor when tested competitively (Jang et al., 1994). This discovery that a synthetic lure can have the same attractiveness as male odor, represents a significant step toward future use of a synthetic pheromonal lure specific for the detection of virgin female medflies. To achieve this goal, the most active coattractants in the minor and intermediate blends (and trace components) must be determined, so that manageable and economical multiple-component formulations can be laboratory and field bioassayed.

The two nonmajor blends enhanced the male odor in a dose- or evaporation rate-dependent manner. The 1-μl capillary tube formulations were optimal for enhancing the pheromone attraction of females. The dose–response data show that the relative enhancement of male odor by both nonmajor blends has apparent quantitative attributes for the minor and intermediate blends, having thresholds (ca. >3.5 μg/hr and >7 μg/hr, respectively), observed maxima (the 1-μl capillary rates of 17 μg/hr and 45 μg/hr), and high dose extinguishing or masking levels (both ca. 1780 μg/hr) (Figure 6).

It is important that the evaporation rates of the two synthetic nonmajor blends that were effective at enhancing male odor attractiveness be compared to rates of emission of the classes of male odor constituents (i.e., major, intermediate, and minor components) released by males. These included the natural male odor (Baker et al., 1985; Heath et al., 1991; Flath et al., 1993), the male odor effluvium of 50 confined-males used in the bioassays reported here, the major component synthetic odor (Jang et al., 1994), and odor from typical male lek aggregations (2–12 males). The natural emission rate is generally high for pheromones and was estimated from the data of Baker et al. (1985) to be 0.16 μg/male/hr, while Heath et al. (1991) reported a maximum of 0.96 μg/hr for three major constituents [(*E*,*E*)-α-farnesene, ethyl (*E*)-3-octenoate, and geranyl acetate]. The five major constituents average 0.82 μg/hr (0.042 and 0.014 μg/hr for intermediate and minor constituents, respectively) and an average overall pheromonal emission of 0.88 μg/male/hr (Flath et al., 1993). The entrained odor of the 50 confined males used in our bioassays might approach 44 μg/hr (i.e., 0.88 μg/hr × 50 males). When this pheromonal effluence was split equally to two cage arenas (ca. 22 μg/hr/cage) with four to eight pheromone-emitting traps in each cage arena, the approximate emission rate per trap was 5.5 to 2.7 μg/hr/trap, respectively. When the usual experimental array of four to six traps per cage was used, the estimated per male odor trap emission rates for the major (ca. 4.3 μg/hr/trap), intermediate (ca. 0.22 μg/hr/trap), and minor (ca. 0.073 μg/hr/trap) constituents were all low relative to the much greater emission rates of the synthetic intermediate and minor blends emitted from the 1-μl capillaries.

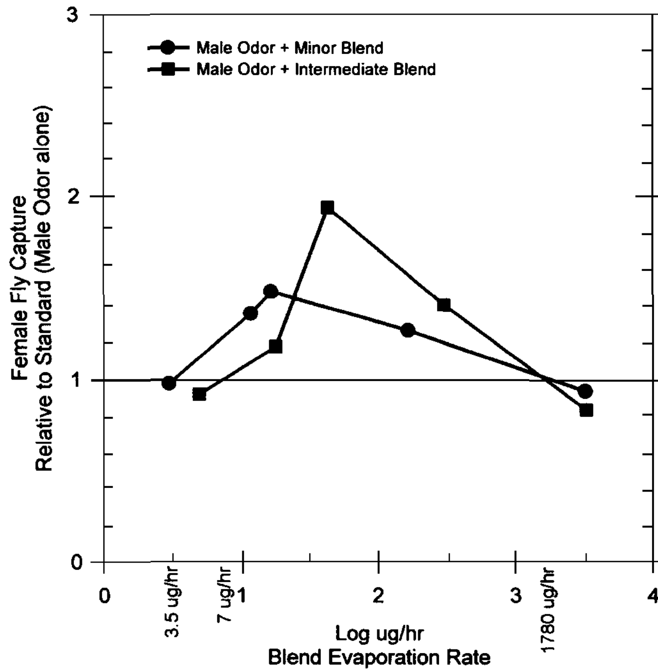

FIG. 6. Dose-dependent enhancement of male odor attractiveness by the intermediate and minor component blends. Capture of virgin female medflies relative to male odor alone (horizontal line at value of 1) versus log evaporation rates of the blend formulations.

The emission of the intermediate blend from a 1-μl capillary (45 μg/hr/trap) was ca. 10× the emission rate per trap of the major constituents' contribution and ca. 200× the emission rate per trap of the natural intermediate constituents' contribution. Similarly, the 1-μl capillary emission of the minor blend (17 μg/hr/trap) was ca. 4× the emission rate per trap of the major constituents' contribution and ca. 230× the emission rate per trap of the natural minor constituents' contribution to male odor. Thus, to enhance the preference and attractiveness of the natural male odor significantly to responding female medflies, both of the synthetic nonmajor blends in our laboratory experiments had to evaporate at much higher rates than their natural homologs in the male odor, thereby creating a distortion in the natural proportions of the constituents.

Previous flight-tunnel bioassays of Jang et al. (1994) demonstrated that the odor generated by five males was 2.9× more attractive to virgin female medflies than the synthetic major components in direct competitive dual-choice tests, even though the combined delivery rate of those five major components (ca. 19.8 μg/hr/trap) is equivalent to the odor emission of major constituents from

not five but 24 males (19.8 μg/hr ÷ 0.82 μg/hr). This analysis emphasizes that full pheromonal attractiveness is not solely based on major constituents, because for the synthetic major components to have any competitive activity, delivery rate had to be greatly increased relative to their presence in the male odor content. This required exaggeration of the delivery rate of the major components to achieve bioactivity suggests that additional, critical, coactive attractant components are missing (i.e., nonmajor components), as defined in Roelofs' (1978) threshold hypothesis for pheromone blend activity. We found that the minor blend enhanced the attractiveness of major components so that it attained parity with the male odor. This is the first demonstration of a synthetic lure rivaling the natural pheromone of medflies.

Shelly et al. (1994) hypothesized that the relatively low mating success of individual male medflies resulted from an inability to pheromonally attract females to their territories. A behavioral adaptation that improves mating success is the lek mating system wherein 2–12 males aggregate on foliage for attracting, courting, and copulating with responding females (Arita and Kanoshiro, 1986, 1989; Shelly et al., 1993, 1994; Whittier et al., 1992, 1994). The entity or source of long-range attraction is the lek, not the individual males. Thus, the lek is a site of pooled resources.

The pheromonal odor of individual males is released in large amounts (at least the major components) and is detectable by the human nose as a malodorous, rank, pungent, "stale mucus" smell (Back and Pemberton, 1918). Human detection of the odor is attributed primarily to our keep perception of one of the major components, 1-pyrroline [defined by Amoore et al. (1975) as the spermous primary odor, and possibly a vestigial human pheromone]. The emitted odor from a medfly lek is of such strong intensity that one can simply use olfaction to find lek sites (Prokopy and Hendrichs, 1979; Hendrichs and Hendrichs, 1990).

Lek aggregations increase the overall quantity of pheromone released by medflies (McDonald, 1987) with perhaps little or no distortion of the qualitative complexity or proportions of the multiple constituents released (Flath et al., 1993). Thus, the increased quantity of pheromone emitted by a lek might achieve a critical emission rate for certain key nonmajor components that are crucial for optimal choice discrimination and attraction of females. These key nonmajor components perhaps act as synergistic coactive attractants with the major components, increasing the pheromonal odor complexity and its attractiveness. The benefit and selective advantage of lekking may be the pooling of a limited resource, which we suggest is the minor or trace components of the pheromone.

Future research needed to develop the potential of a pheromonal lure for medfly females and to exploit our findings includes: (1) defining individual active nonmajor components for female attraction, (2) developing a slow releas-

ing lure formulation and an effective trap design for females, (3) investigating male responses to pheromone lek formation (Shelly et al., 1993) and lek recruitment, (4) improving the success of SIT by selective breeding of males that produce greater and more optimal proportions of critical nonmajor pheromonal components, and (5) possibly treating SIT male medflies (through ingestion or topical application) with particular nonmajor pheromonal components (or precursors/analogs) so as to increase their release in pheromone calling.

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ETHANOL AND WATER IN *Pseudotsuga menziesii* AND *Pinus ponderosa* STUMPS

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Abstract—Douglas fir (*Pseudotsuga menziesii*), west of the Oregon Cascades, and ponderosa pine (*Pinus ponderosa*), east of the Cascades, were cut during the fall in conjunction with various forest management practices. Trees cut varied in size and age, and the stumps were exposed to disparate winter temperatures and precipitation patterns. Nevertheless, the stumps showed similar responses in their synthesis and accumulation of ethanol. The following spring, ethanol concentrations in above-ground tissues of both species ranged from 3 to 116 times higher than in their corresponding root tissues. We suggest that this difference results from the above-ground tissues being more hypoxic than roots because they were exposed to more water from precipitation and warmer temperatures. Ethanol concentrations in the above-ground tissues of ponderosa pine stumps were about two to six times higher than in Douglas fir, and root tissues from pine stumps usually contained more ethanol after anaerobic incubation than roots from Douglas fir. Ethanol and volatile terpenes released from stumps can attract various beetle species that not only vector root diseases, but can also damage or kill seedlings and saplings. Understanding the dynamics of ethanol synthesis and accumulation in stumps and slash might contribute to new alternatives for managing these insects.

Key Words—Douglas fir, ponderosa pine, anaerobic respiration, fermentation, kairomone.

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INTRODUCTION

Stumps and other woody debris left after harvesting or thinning a forest can attract various insect species that may vector diseases and also damage or kill seedling and regenerating saplings. Examples include the pine weevil (*Hylobius abietis* L.) in Europe (Nordlander, 1987; Wilson et al., 1996), pales weevil (*H. pales* Herbst) in central and eastern North America (Salom, 1997), the bark beetle *Hylastes nigrinis* (Mannerheim) and weevil *Steremnius carinatus* (Boheman) in western North America, and the bark beetles *Hylastes ater* Paykull and *Hylurgus ligniperda* F. in South America (Cielsa, 1988). *Hylobius pales* vectors spores of *Leptographium procerum* (Kendrick) M.J. Wingfield, the causal agent of procerum root disease (Nevill and Alexander, 1992a–c), while *Hylastes nigrinis* (Mannerheim) and *Steremnius carinatus* (Boheman) vector spores of *Leptographium wageneri* (Kendr.) Wingf., the causal agent of black-stain root disease (Harrington et al., 1985; Bedard et al., 1990; Witcosky et al., 1986a,b).

Many of the insects that colonize stumps, slash, or roots are attracted to artificial sources of ethanol, α-pinene, mixtures of host terpenes or crude oleoresins, or binary combinations of these compounds with ethanol (Nordlander et al., 1986; Nordlander, 1987; Schroeder and Lindelöw, 1989; Witcosky et al., 1987; Bedard et al., 1990; Rieske and Raffa, 1991; Lindelöw et al., 1993; Hoffman et al., 1997). Freshly cut stumps and slash release substantial quantities of oleoresin volatiles and may also produce ethanol. Logs from Douglas fir [*Pseudotsuga menziesii* (Mirb.) Franco], western hemlock [*Tsuga heterophylla* (Raf.) Sarg.], and western redcedar [*Thuja plicata*, Donn ex D. Don), all synthesized and accumulated ethanol when left in the forest through winter (Kelsey, 1994a,b; Kelsey and Joseph, 1997). In Sweden, stumps of Scotch pine (*Pinus sylvestris* L.) and Norway spruce [*Picea abies* (L.) Karst.] cut in April and colonized by *Hylobius abietis* contained ethanol when sampled from May through August of the same year (von Sydow and Birgersson, 1997). Douglas fir or ponderosa pine, *Pinus ponderosa* Dougl. ex Laws., infected with black-stain or other root diseases may contain high ethanol concentrations in roots or stem tissues near the root collar (Kelsey and Joseph, 1998; Kelsey et al., 1998a). Severe injury to the stems of Scotch pine in the fall, by removing their tops at 6 m above ground or by girdling the phloem at 1 m, resulted in ethanol accumulation in stem tissues the following spring (Sjödin et al., 1989).

Although ethanol is not the only volatile to attract forest insects, it certainly plays an important role in host selection for the less aggressive, secondary species that attack and colonize diseased or dying forest woody debris. In this paper we report the spring ethanol concentrations in stumps of Douglas fir and pondeosa pine cut the previous fall under a variety of forest management practices and discuss the environmental conditions that may have influenced ethanol synthesis.

METHODS AND MATERIALS

General Sampling Procedures. All tissues were sampled with a 5-mm-ID increment bore. The phloem and sapwood were separated and sealed in glass, screwcap vials (45 × 15 mm OD) with Teflon-lined caps, and immediately frozen on Dry Ice. In the laboratory they were stored at −36°C and analyzed the same day or the day after collecting. Root samples subjected to anaerobic conditions were placed on ice during transport, because freezing with Dry Ice can deactivate the enzymes.

Douglas Fir Stumps. On May 16, 1994, 10 stumps and 10 adjacent live trees were sampled at the McDonald Experimental Forest near Corvallis, Oregon (Table 1). Cores were taken between soil level and mid-stump height, at the cardinal directions. North–south and east–west cores were pooled by tissue type. The sapwood was sampled to a depth of 1.0 cm.

Another four stumps of Douglas fir were each sampled on March 7 and 14, 1996, from the Dunn and McDonald Experimental forests, respectively, near Corvallis, Oregon (Table 1). Three cores were taken from each stump along a vertical transect positioned above a major lateral root. The top core was 6–12 cm below the cut surface, the middle core was at the root collar near soil level, and the bottom core was 20–50 cm down from the root collar on top of an excavated root. The sapwood was sampled to a 6-cm depth and divided into three consecutive segments of 0–2, 2–4, and 4–6 cm for separate analysis.

Ponderosa Pine Stumps. On April 9, 1996, six ponderosa pine stumps were sampled near the town of Sisters, Oregon, and on May 1, four stumps were sampled at Gilchrist, Oregon (Table 1). Cores were taken from three locations around each stump. At each location one core was taken from 3 to 6 cm below the cut surface and another was taken directly below this from a root under the soil. These stumps were shorter than Douglas fir and their cut surface closer to the soil, so samples were not taken at soil level. The sapwood was sampled to 6 cm depth and divided into two pieces, 0–3 and 3–6 cm for separate analysis.

Anaerobically Induced Ethanol in Roots. Since ethanol concentrations in root tissues were much lower than in stump tissues below the cut surface, we wanted to know whether the root tissues would produce ethanol when subjected to anaerobic conditions. To address this question, on May 1, 1996 one additional core from a lateral root or taproot was collected from four of the small stumps west of Sisters and two of the large stumps at Gilchrist. These were a subset of the pine stumps sampled for constitutive ethanol concentrations. Similarly, on May 6, cores were taken from roots of two stumps from the Dunn and two from the McDonald Forest sites previously sampled in March. The phloem and sapwood were separated and the sapwood divided into two pieces, 0–3 and 3–6 cm depth, for separate analysis.

Each sample was weighed in a preweighed autosampler vial (22 × 75 mm

TABLE 1. SITE AND STUMP CHARACTERISTICS OF DOUGLAS FIR AND PONDEROSA PINE SAMPLED FOR ETHANOL ANALYSIS IN YEAR FOLLOWING HARVEST[a]

	Douglas fir			Ponderosa pine	
	McDonald	McDonald	Dunn	Sisters	Gilchrist
Cutting period	Oct, 93	Oct–Dec, 95	Oct, 95	Oct–Dec, 95	Nov, 95
Constitutive ethanol sample date	May 16, 94	March 14, 96	March 7, 96	April 9, 96	May 1, 96
Root sample date for anaerobic ethanol		May 6, 96	May 6, 96	May 1, 96	May 1, 96
Harvest method	Commercial thin	Commercial thin	Clear cut	Precommercial thin	Selection cut
Elevation (m)	378	122	366	1220	1281
Location	44°39′N 123°16′W	44°40′N 123°14′W	44°41′N 123°18′W	44°14′N 121°30′W	43°29′N 121°41′W
Tree age (yr)	60–65	55-60	100–120	10–15	200–300
Stump diam. (cm)	60.0	42.0	69.0	15.0	75.0
Mean daily min. temperature (°C)	4.3	3.7	3.7	−2.5	−1.9
Mean daily max. temperature (°C)	13.3	13.7	13.7	10.7	10.7
Precipitation (cm)	102.4	198.6	198.6	40.8	27.5

[a]Weather data were collected from the nearest weather stations. Corvallis Water Bureau data (elev. 180 m) was used for the McDonald and Dunn sites. The Sisters Ranger station provided data for the Sisters site (elev. 970 m), and the Bend weather station (elev. 1116 m) was used for the Gilchrist site. The mean temperatures and total rainfall was calculated from October to May except for the McDonald and Dunn Forests, where it was calculated from October to March.

OD) and sealed with a septum. Vials were purged for 30 sec with N_2 by inserting inlet and outlet needles through the septa. Purged vials were incubated at 30°C in the dark for 24 hr to induce ethanol synthesis. After incubation they were heated for 30 min at 102°C to deactivate enzymes and then analyzed by headspace gas chromatography. Vials were decapped and heated at 102°C for 16 hr, desiccated for 30 min, and weighed to obtain tissue water contents and dry weights.

Ethanol Analysis. Ethanol was analyzed by a multiple headspace extraction procedure using a Perkin Elmer HS40 autosampler and Hewlett Packard 5890 gas chromatography with a J&W Scientific DB-Wax column. Operating conditions and settings were the same as reported previously (Kelsey and Joseph, 1998). The column oven was set isothermally at 50°C for Douglas fir tissue, whereas for ponderosa pine samples it was set at 50°C for 1 min, then increased to 80°C (20°C/min) and held for 1.5 min. This prevented carryover interference from monoterpenes between samples, which was more of a problem for ponderosa pine than Douglas fir. The oven program has no effect on ethanol quantitation. Since ethanol is readily soluble in water, and insects attack stumps while they have a relatively high water content, we calculated the ethanol concentrations on a fresh weight basis, rather than dry weight so they more accurately represent the concentrations encountered by insects.

Statistical Analysis. All analyses were made using the PROC MIXED procedure of SAS (SAS Institute, 1996). Differences in ethanol concentrations or water contents between the phloem and sapwood of live trees and stumps were analyzed as a split-plot with treatment (tree or stump) as the main effect and tissue as the subplot. For stumps at all other sites, differences in ethanol concentrations or water content were analyzed as a strip-plot with vertical position on the stump and radial depth (phloem and sapwood segments) as strips (Petersen, 1985). Depth samples were analyzed as a repeated measure because they were not independent (Ramsey and Schafer, 1997). Means and standard errors for the anaerobically induced ethanol concentrations in Table 2 were calculated separately for each species and site and were not compared by statistical analysis. Where necessary, data were natural log transformed to meet homogeneity of variance and normality assumptions. Back-transformed means are presented for transformed data. Significant differences between means were separated using Fisher's protected LSD at $\alpha = 0.05$.

RESULTS

Constitutive Ethanol and Water in Douglas Fir Stumps. Ethanol concentrations in stumps and adjacent trees in the spring of 1994 were dependent on treatment (stump or tree) and tissue type (Figure 1A). For trees, ethanol concentrations in the phloem were higher than sapwood ($P \leq 0.001$), whereas in

TABLE 2. CONSTITUTIVE (N = 12–36) AND N_2-Induced (N = 2–8) ETHANOL CONCENTRATIONS (MEAN ± SE) IN PHLOEM AND SAPWOOD FROM ROOTS OF DOUGLAS FIR AND PONDEROSA PINE STUMPS

	Ethanol (μmol/g fresh wt)			
	Phloem		Sapwood	
Species/site	Constitutive	N_2-induced	Constitutive	N_2-induced
Douglas fir				
McDonald	0.07 ± 0.01	25.89 ± 0.86	0.34 ± 0.12	2.78 ± 1.76
Dunn	0.35 ± 0.06	18.35 ± 13.35	1.02 ± 0.30	2.37 ± 1.31
Ponderosa pine				
Sisters	0.52 ± 0.10	141.16 ± 24.08	0.18 ± 0.02	13.07 ± 2.53
Gilchrist	2.33 ± 2.08	16.55[a]	0.87 ± 0.29	4.63 ± 0.23

[a]On these large stumps the phloem was thin, only one sample was available from this site.

stumps the opposite was observed ($P < 0.001$). Stumps and trees had similar ethanol concentrations in their phloem, while stumps had 35 times more sapwood ethanol than trees ($P < 0.001$). Water contents were also dependent on the treatment and tissue type (Figure 1B). In stumps and trees the phloem contained more water than the sapwood (both $P < 0.001$). Phloem from stumps had a higher water content than phloem from trees ($P < 0.001$), whereas the sapwood water content did not differ between stumps and trees ($P = 0.245$).

In the spring of 1996, ethanol concentrations in overwintering Douglas fir stumps from the Dunn Forest were dependent only on their vertical position, with no differences between radial depths (Figure 2A). The ethanol concentrations were significantly different between all three vertical positions ($P \leq 0.026$). A similar trend was observed among vertical positions in stumps from the McDonald Forest (Figure 2C), but it was not significantly different ($P = 0.063$).

The water content in Douglas fir stumps from both forests was dependent on the radial depth and not the position (Figure 2B and D). Phloem always contained a much higher water content than sapwood ($P < 0.001$ for both forests). The sapwood segment nearest the phloem had the highest water content, and it decreased with each 2-cm radial depth (S1 > S2 > S3, averaged across positions). These differences were all significant (all $P \leq 0.028$) in Dunn Forest stumps. In McDonald Forest stumps the differences were significant for segments S1 and S3 ($P = 0.004$), S2 and S3 ($P = 0.056$), but not for S1 and S2 ($P = 0.073$).

Constitutive Ethanol and Water in Ponderosa Pine Stumps. Ethanol concentrations in small ponderosa pine stumps near Sisters were dependent on the radial depth and position (Figure 3A). At each radial depth, ethanol concentrations near the cut surface were substantially greater than the concentrations in the roots (all $P < 0.001$). Near the cut surface, ethanol concentrations among

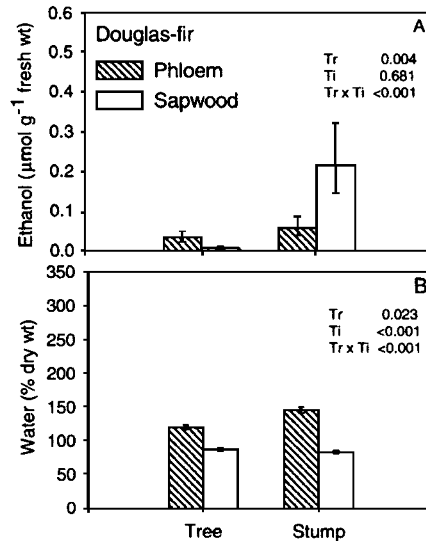

FIG. 1. Ethanol concentrations (A) and water contents (B) in the phloem and sapwood from trees and stumps of Douglas fir, May 16, 1994. Values are means ± SE. Insert lists P values from the ANOVA, Tr = treatment (tree or stump), Ti = tissue type.

depths were different between S1 and S2 (P = 0.013), but not P and S1 (P = 0.907), or P and S2 (P = 0.148). In roots, phloem ethanol concentrations were greater than either the S1 (P = 0.002) or S2 (P = 0.010) sapwood concentrations, which were similar (P = 0.325).

In large ponderosa pine stumps from Gilchrist the ethanol concentrations were dependent on both the position and radial depth (Figure 3C). At each radial depth, ethanol concentrations near the cut surface were substantially greater than at the corresponding depth in roots (all $P \leq 0.005$). Near the cut surface, ethanol concentrations were highest in the phloem and decreased with each incremental depth of sapwood (all $P \leq 0.033$). In roots, there were no differences in ethanol concentrations among depths (P, S1, or S2; all P = 0.370).

Water content for the stumps at Sisters was dependent on the position and radial depth (Figure 3B). Phloem had a much higher water content than either the S1 or S2 sapwood segments (all $P \leq 0.001$), regardless of the position. The S1 and S2 segments had similar water contents at each position (P = 0.279). For stumps at Gilchrist, the tissue water content was dependent on position or radial depth, but not their interaction (Figure 3D). Water was more abundant in tissues near the cut surface than in tissues from the roots ($P \leq 0.025$), and phloem always had a much higher water content than the S1 and S2 sapwood segments (both $P < 0.001$). S1 and S2 had similar quantities of water (P = 0.674).

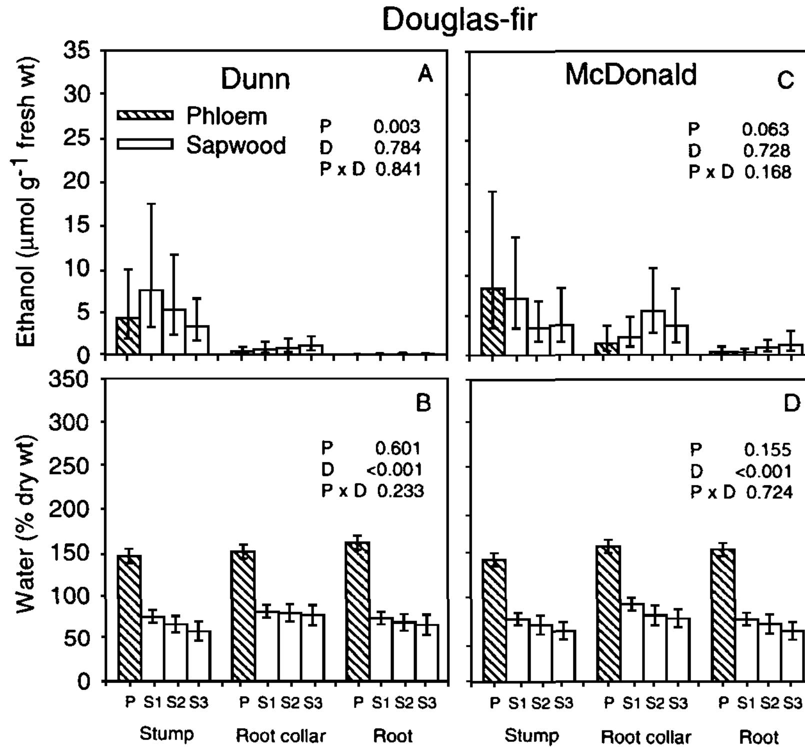

FIG. 2. Ethanol concentrations (A and C) and water contents (B and D) in the phloem and sapwood of Douglas fir stumps from the Dunn (A and B) and McDonald (C and D) forests. Increment cores were taken at three positions along a vertical transect (in the residual stem 8–12 cm below the cut surface, near the root collar at soil level, and from an excavated root 20–50 cm below the root collar). Each core was divided into four consecutive radial segments (P = phloem; S = sapwood; S1 = 0–2 cm; S2 = 2–4 cm; S3 = 4–6 cm). Values are means ± SE. Insert lists *P* values from the ANOVA, P = vertical position, D = radial depth. Stumps were sampled March 7 and 14, 1996, from the Dunn and McDonald forests, respectively.

Anaerobic Ethanol Concentrations in Root Tissues. After 24 hr of anaerobic incubation, the root tissues from stumps of both species contained substantially higher ethanol concentrations than the constitutive quantities measured on the previous sampling date (Table 2). For both species, these differences were largest in the phloem, with concentrations ranging from 7 to 370 times higher after incubation. Increases in the sapwood were less dramatic, ranging from 2 to 73 times higher after incubation. The anaerobic ethanol concentrations in the tissues of ponderosa pine stumps were two to eight times greater than in the

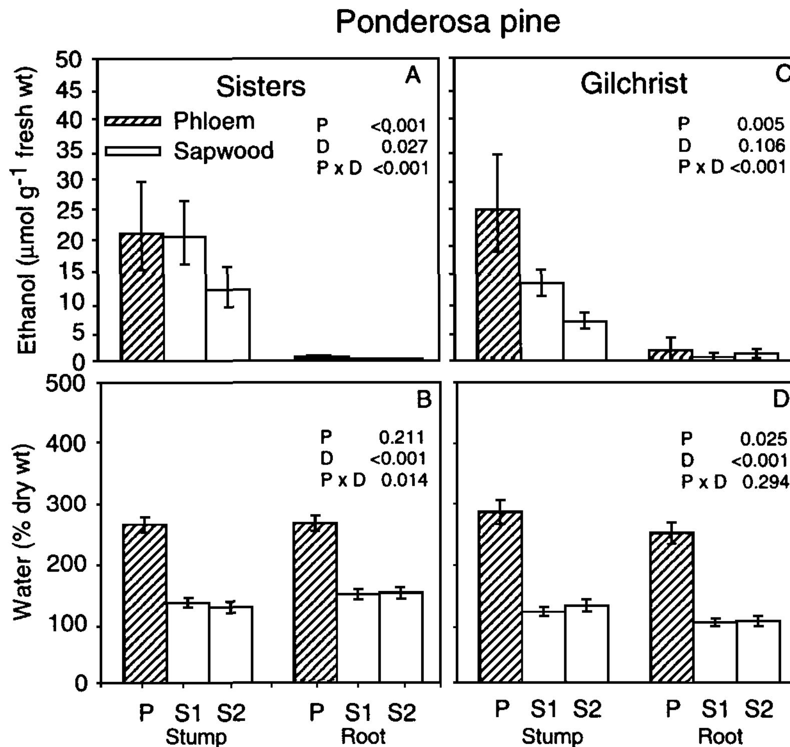

FIG. 3. Ethanol concentrations (A and C) and water contents (B and D) in the phloem and sapwood from stumps of ponderosa pine near the towns of Sisters (A and B) and Gilchrist (C and D), Oregon. Increment cores were taken at two positions along a vertical transect (in the residual stem 3 to 6 cm below the cut surface, and from a root below the soil). Each core was divided into three consecutive radial segments (P = phloem; S = sapwood: S1 = 0–3 cm; S2 = 3–6 cm). Values are means ± SE. Insert lists *P* values from the ANOVA, P = vertical position, D = radial depth. Stumps were sampled April 9, and May 1, 1996, near Sisters and Gilchrist, respectively.

same tissues of Douglas fir stumps, with the exception of phloem from ponderosa pine at Gilchrist, which had ethanol concentrations similar to those in the phloem from Douglas fir.

DISCUSSION

Fall-cut stumps demonstrated similar responses in accumulation and distribution of ethanol in their tissues despite different species, stump diameters, ages

of trees cut, silvicultural treatments, geographic sites, and weather conditions. In spring, the highest ethanol concentrations always occured in the residual stem tissues near the cut surface, with very low concentrations in the roots. Ethanol was probably synthesized in the stem tissues and not transported from below ground because the concentration gradient decreased toward the roots. If the ethanol had been synthesized in roots and moved upward, as it does when trees were flooded (Joseph and Kelsey 1997), the gradient would have been in the opposite direction (MacDonald and Kimmerer, 1991). Although roots contained low constitutive ethanol concentrations compared to the above-ground tissues, they were still capable of synthesizing high ethanol concentrations when incubated under anaerobic conditions. This demonstrates the roots had not previously exhausted their ability to synthesize ethanol, and the ethanol then dissipated. Since all live tissues in Douglas fir and ponderosa pine quickly synthesize ethanol when subjected to anaerobic conditions (Kelsey et al., 1998b), it is reasonable to assume that the above-ground tissues in stumps may have synthesized more ethanol because they were exposed to more hypoxia or anoxia than in roots.

There is evidence to suggest that precipitation and tissue temperatures may contribute to the dissimilar ethanol concentrations in the stem and roots of stumps. Douglas fir logs cut in October and left exposed to rain through winter contained significantly higher ethanol concentrations than dry logs protected from rain (Kelsey and Joseph, 1999). It appears that rain absorbed by the outer bark creates a barrier to gas exchange with the atmosphere, and if it remains long enough for internal O_2 levels to decline, the tissues may become hypoxic and initiate ethanol synthesis. Rain or melting snow could create a similar water barrier near the surface of stumps and interfere with gas exchange in the tissues below. Since precipitation does not directly contact roots buried in the soil, it might interfere less frequently with their gas exchange than in above-ground tissues. Gas exchange in roots may not be impacted until the soil is nearly saturated. Furthermore, as tissue temperatures rise, the metabolic rates will increase, causing aerobic respiration to consume O_2 and generate CO_2 more rapidly than at lower temperatures (Sprugel et al., 1995). Warm tissues with impaired gas exchange will become hypoxic faster than cool tissues (unpublished data). Above-ground stump tissues are more exposed to warm sunlight than roots protected by the cool, thermally buffered soil. In spring, a combination of precipitation and warm temperatures would provide optimum conditions for generating hypoxia and ethanol synthesis in above-ground stump tissues, but not in the roots.

While precipitation may play a role in creating tissue hypoxia in stumps, a measurable increase in water content is not necessary for ethanol synthesis to occur. For example, at all sites except Gilchrist, the stump tissues near the cut surface had the same water content as roots, but their ethanol concentrations were very different. Similarly, the sapwood of stumps and trees sampled in 1994

had the same water content, but stumps had much higher ethanol concentrations. Moreover, the tissue water contents in Douglas fir logs exposed to rain through winter were the same as in logs protected from rain, but ethanol concentrations were significantly higher in logs exposed to rain (Kelsey and Joseph, 1999).

Constitutive ethanol concentrations in above-ground tissues of ponderosa pine were two to six times higher than in similar tissues of Douglas fir, and root tissues from pine typically synthesized more anaerobic ethanol than roots of Douglas fir. This is consistent with our previous observations where needles, roots, phloem, and sapwood from ponderosa pine seedlings produced more ethanol than Douglas fir seedlings when fertilized with or without N (Kelsey et al., 1998b). Furthermore, stems from seedlings of several pine species, including ponderosa, all produced more ethanol than stems of Douglas fir when subjected to anaerobic conditions (Kelsey, 1996). Ponderosa pine appears to have an inherent ability to produce higher ethanol concentrations than Douglas fir.

Ethanol concentrations in the 1994 stumps were lower than in 1996 stumps in part because they were sampled about halfway between the cut surface and soil level where concentrations are lower than near the cut surface. Furthermore, these were the only stumps in this study that had been attacked by Douglas fir bark beetles (*Dendroctonus pseudotsugae* Hopkins) and ambrosia beetles [*Trypodendron lineatum* (Oliver) or *Gnathotrichus* spp.], prior to sampling. Some ethanol may have been lost by venting from the beetle galleries, or it could have been metabolized by microbes growing in the galleries. Root pathogens such as *Leptographium wageneri*, the fungus causing black-stain root disease in conifers, and *Armellaria mellea* (Vahl.:Fr.) Kummer, can use ethanol as a carbon and energy source for growth (Weinhold and Garraway, 1966; authors unpublished data for *L. wageneri*). Similarly, microbes colonizing insect galleries may metabolize ethanol, but this remains to be demonstrated. Sapwood from the 1994 stumps had much higher ethanol concentrations than sapwood in live trees, whereas there were no differences in their phloem ethanol concentrations. Here again, colonization by Douglas fir beetles could have contributed to lower ethanol concentrations in the phloem from stumps.

In a Michigan Scotch pine plantation, stumps with living branches attached were not attacked by *Hylobius pales*, whereas adjacent stumps without branches were colonized (Corneil and Wilson, 1984). Our results might help explain this response. Branchless Scotch pine stumps probably synthesized ethanol which attracted *H. pales*. In contrast, stumps with branches attached remained alive with minimal ethanol synthesis, as in healthy trees (von Sydow and Birgesson, 1997; Kelsey and Joseph, 1998), and therefore were unattractive to the weevils. Volatile terpenes were probably not a factor in this selection because the stumps were similar in size and would release comparable quantities of these compounds. In some eastern states *H. pales* is managed by cutting stumps close to the ground and covering them with soil (Salom, 1997). This could reduce

stump ethanol concentrations in two ways. First, it minimizes the volume of above-ground tissues where ethanol concentrations are high in the spring. Second, covering the remaining stump with soil may limit the tissues' ability to synthesize ethanol because they are now subjected to the same environment that may minimize ethanol synthesis in roots. However, as soil temperatures increase, the buried tissues might begin to accumulate ethanol. For instance, roots (80 cm below the cut surface) on stumps of Norway spruce or Scotch pine cut in April contained ethanol when sampled during the summer months of the same year (von Sydow and Birgesson, 1997).

Various external and internal environmental factors influence the synthesis, metabolism, and accumulation of ethanol in dying woody tissues, thus causing great temporal and spacial variation in ethanol concentrations (Sjödin et al., 1989; MacDonald and Kimmerer, 1991; Kelsey 1994a,b; Kelsey and Joseph 1997; Kelsey et al., 1998b). This variability will subsequently affect the response of insects that utilize ethanol as a kairomone. A better understanding of ethanol synthesis in stumps and slash and how it is affected by environmental factors might help determine optimum times for thinning or contribute to the development of new treatments for these residues to minimize their attractiveness to unwanted forest insects. With increasing emphasis on thinning and selective harvesting in forest management, and with greater quantities of woody debris of various sizes being left in forests for wildlife habitat, nutrient cycling, and soil amendments, it is important that these residues do not subject the remaining trees to increased risks from insects and disease.

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ETHANOL AND AMBROSIA BEETLES IN DOUGLAS FIR LOGS EXPOSED OR PROTECTED FROM RAIN

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Abstract—Logs from the base of Douglas fir (*Pseudotsuga menziesii*) trees cut in October 1993 were randomly assigned to one of three treatment groups: (1) wet logs—cut from the fallen tree and left exposed to rain, (2) dry logs—cut from the fallen tree, placed on blocks, and protected from rain under a plastic tent, and (3) crown logs—left attached to the fallen tree with its branches intact and exposed to rain. The following May, ethanol concentrations were highest in the phloem and sapwood of wet logs (0.24 and 0.35 μmol/g fresh wt, respectively). Ethanol concentrations in tissues from dry and crown logs were similar to each other (ranging from 0.002 to 0.03 μmol/g fresh wt), but were significantly lower than in wet logs. It appears that rain absorbed by the outer bark of wet logs creates a barrier to gas exchange between living tissues and the atmosphere, which facilitates the development of hypoxic conditions necessary for ethanol synthesis and accumulation. Branches on crown logs exposed to rain help maintain low ethanol concentrations in the log tissues; we discuss several potential mechanisms to explain this response. By early September, the densities of *Gnathothrichus* spp. gallery entrance holes were high on wet logs (21.5/m^2) and low on dry (2.5/m^2) and crown logs (5.8/m^2), indicating their preference for logs with higher ethanol concentrations. Protecting logs from rain will significantly reduce ethanol concentrations and the density of ambrosia beetle galleries. Leaving branches attached to logs will produce similar results, but its effectiveness may vary depending on the environmental conditions. Host selection by secondary scolytid beetles that use ethanol as a kairomone can be manipulated and possibly managed by controlling the production of ethanol in the host resource.

Key Words—*Pseudotsuga menziesii*, *Gnathothrichus* spp., ethanol, host selection, anaerobic respiration, fermentation, Coleoptera, Scolytidae.

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INTRODUCTION

Insects that colonize dying or recently dead coarse woody debris are ecologically important in initiating decomposition and nutrient cycling, thus promoting long-term productivity and stability of forest ecosystems (Harmon et al., 1986; Schowalter et al., 1992; Schowalter and Filip, 1993). However, these insects can also become pests of economic importance. For example, ambrosia beetles are detritivores, but their attacks can greatly decrease the value of commercial logs or lumber (McLean, 1985). Bark beetles and weevils can cause damage to seedling and sapling regeneration or can vector spores of pathogens from diseased stumps to nearby healthy trees (Harrington et al., 1985; Witcosky et al., 1986a,b; Ciesla, 1988; Bedard et al., 1990; Nevill and Alexander, 1992a–c). Various forms of woody residues are being retained in forests in greater quantities than in the past to maintain and facilitate important ecological processes and functions (Schowalter and Filip, 1993). Stand thinning is being used more frequently to achieve desired vegetative patterns and management objectives, but it may also provide woody residues that can aggravate insect and disease problems.

Tissues in dying or recently dead woody residues will typically synthesize and accumulate some ethanol as they age (Moeck, 1970; Sjödin et al., 1989; MacDonald and Kimmerer, 1991; Kelsey, 1994a,b; von Sydow and Birgersson, 1997; Kelsey and Joseph, 1999). If it escapes into the atmosphere, ethanol can function as a primary host attractant for various insect species that colonize and reproduce in the woody substrate (Moeck, 1970; Klimetzek et al., 1986; Nordlander et al., 1986; Witcosky et al., 1987; Chénier and Philogène, 1989; Liu and McLean, 1989). For many conifers, the simultaneous release of α-pinene, or mixtures of their terpenes, and ethanol may further enhance or synergize insect responses compared to ethanol alone (Tilles et al., 1986; Nordlander, 1987; Raffa and Hunt, 1988; Chénier and Philogène, 1989; Schroeder and Lindelöw, 1989; Phillips, 1990; Lindelöw et al., 1993). If the insect produces a pheromone, ethanol may also enhance its attractiveness, with or without the presence of α-pinene (Pitman et al., 1975; Borden et al., 1980; Shore and McLean, 1983; Liu and McLean, 1989).

Ethanol accumulation in woody tissues is dependent on the rates of synthesis, movement, and possibly metabolism into other compounds when the supply of O_2 is adequate (MacDonald and Kimmerer, 1993). Each of these processes may be influenced by internal and external factors, thus causing the amount of ethanol in woody debris to vary significantly. Under similar environmental conditions ethanol concentrations may vary among tree species or among positions within logs (Kelsey 1996; Kelsey and Joseph, 1997). When branches remain attached to logs, the ethanol concentrations will be lower than in branchless logs (Kelsey, 1994b). Season of harvest can affect subsequent ethanol concen-

trations. For example, logs cut in November produced more ethanol in their first two months after harvest than logs cut in January (Kelsey, 1994a).

Since ethanol is a primary attractant or kairomone for the various species of bark and ambrosia beetles described above, a better understanding of the factors regulating ethanol accumulation in woody tissues could lead to alternative management strategies to help mitigate undesirable behavior of these insects. In a previous experiment, rain was identified as an environmental factor that could potentially influence ethanol synthesis and accumulation in logs (Kelsey, 1994a), but it required further verification. The objectives of this study were to determine whether exposure to rain affects ethanol concentrations in aging log tissues and whether the ethanol concentrations influence which logs ambrosia beetles will select and colonize as a host.

METHODS AND MATERIALS

Study Site. The study was located in the McDonald Experimental Forest (Oregon State University) near Corvallis, Oregon (44°39′N; 123°16′W), in a Douglas fir [*Pseudotsuga menziesii* (Mirb.) Franco] stand on the northwest side of a ridge at 1240 m elevation and slope ranging from 15 to 32%. This area had been harvested in the late 1920s or early 1930s, replanted with Douglas fir in 1936, and thinned in the 1970s and 1980s. Our study was set up during a commercial thinning in September and October 1993. After thinning, there were 203 dominant or codominant Douglas-fir per hectare with a diameter of 25.4 cm or greater, with 55% having a diameter of 40–56 cm. Canopy closure was estimated at 78%.

Treatments. Eight blocks were located along a 200-m line, with variable distances between blocks. Three trees per block were cut on October 20, 1993. Ends of logs were examined and any portion with damaged or missing bark in the first 2 m was removed. Each log was tagged and randomly assigned to one of three treatments: (1) wet log—a log cut from the base of a felled crown and left exposed to rain through winter; (2) dry log—a log cut from the base of a felled crown and placed under a plastic tent through winter to protect from rain; and (3) crown log—a log that remained attached to the base of a felled crown (with branches intact) and exposed to rain through winter. Wet and dry logs (183 cm in length) were removed from the base of cut trees and repositioned close to the unmovable crown logs. They were placed lengthwise along the contour with their top side level. Most crown logs were also perpendicular to the slope. Within blocks, the final distance between logs varied from 2 to 10 m. Unused crown portions were removed from the site.

Dry logs were placed on wooden blocks to eliminate direct contact with the soil and a plastic tent was constructed over them on October 26 or 27, 1993.

Two rectangular frames (3.66 × 1.52 m) made with 2.54 m (outside diam.) plastic PVC pipe were bound together with plastic bands and positioned lengthwise over the log as an A-frame. A sheet of clear plastic (6 mil) was stretched over the frame and secured with waterproof tape. On both sides of the tent there was a 30-cm opening between the bottom edge of the plastic sheet and the frame to allow for air movement along the log sides. The tent extended 92 cm beyond each end of the log so that air or insects could enter, yet the logs remained dry. Tents were staked to the ground and left in place through winter. They were removed on May 6, 1994, three days after the first ambrosia beetle holes were found in a wet log. Tents were replaced during periods of rain from May 15–18, May 31–June 9, June 13–17, and then removed permanently on June 17. Temperatures in the tents were not measured, but probably did not increase much above ambient because the tents were shaded under the canopy and received limited solar radiation with a northwest exposure.

Rainfall Measurements. On November 8, 1993, one rain gauge (15.5 cm diam. and 16.5 cm deep) was placed in each block near the wet log treatment. A small amount of oil was placed in each gauge to float over the water and minimize evaporation. The water was collected periodically, measured with a graduated cylinder, and converted into centimeters of rainfall.

Analysis of Volatiles and Water. Log tissues were sampled twice during the experiment to measure ethanol concentrations and water contents. Samples were first collected on May 24 and 25, 1994, three weeks after the initial ambrosia beetle gallery holes were observed in a wet log. The second set of samples was taken on August 25, 1994, about two weeks before the densities of ambrosia beetle galleries were counted. Three cores were taken along the top of each log with an increment borer (5 mm diam.), one at 36–40 cm from each end, or the equivalent distance on crown logs, and another at the log center. Phloem was separated from the sapwood (1.0 cm depth) and the three pieces of each tissue type combined in a sealed vial (13 × 45 mm) as a single sample. This was repeated along both sides about midway between the log top and soil level, yielding three samples of phloem and three samples of sapwood per log. Vials were frozen with Dry Ice, returned to the laboratory, and stored at −36°C until analyzed. Core holes were plugged with corks. August cores were taken at a distance of 4–6 cm from previous holes.

Ethanol and α-pinene were analyzed as previously described (Kelsey, 1994b; Kelsey and Joseph, 1998) by a multiple headspace extraction procedure with a Perkin Elmer HS40 headspace autosampler connected to a Hewlett Packard 5890 gas chromatograph. Ethanol was quantified with a standard diluted in water. α-Pinene was quantified in May tissue samples only, using a standard diluted in methanol. After analysis, the vials were uncapped and samples dired at 102°C for 16 hr, desiccated for 30 min, and weighed to determine dry weights and water content.

Carbohydrate Analysis. Samples of log tissues were collected on October 25 and 26, prior to constructing the tents, and then again on May 26 and 27 to determine concentrations of starch and soluble sugars. One core (19 mm wide) was taken about 31 cm from the cut ends, or the equivalent distance on crown logs with a specially adapted bit connected to a power drill. Phloem and sapwood (1.0 cm depth) were separated and each tissue type combined in plastic bags, frozen with Dry Ice, and stored at −36°C until analyzed. Core holes were plugged with corks. May samples for carbohydrates were taken near the cores removed for ethanol analysis.

Concentrations of starch and soluble sugars were measured for duplicate samples of ground (60 mesh) and dried (100°C) tissues. Soluble sugars were extracted with methanol–chloroform–water (2 : 5 : 3) (Haissig and Dickson, 1979; Rose et al., 1991), then measured colorimetrically (Blakeney and Mutton, 1980) after hydrolyzing sucrose. The extracted tissue was redried, the starch hydrolyzed enzymatically, and then quantified colorimetrically as described by Rose et al. (1991, enzyme method 2). Additional details for this procedure are presented in Kelsey et al. (1998).

Densities of Ambrosia Beetle Gallery Holes. From September 7 to 9 bark was peeled from logs and the entrance holes to ambrosia beetle galleries in the sapwood counted in a 25- × 128-cm quadrat positioned lengthwise along the log top starting 30 cm in from the base end. A similar quadrat was positioned and counted on each of the two sides. The diameter of each gallery entrance hole was checked with the end of a No. 53 wire gauge drill bit to separate *Trypodendron lineatum* galleries from the *Gnathotrichus* spp. galleries (Kinghorn, 1957).

Statistical Analysis. The data were analyzed with SAS software (SAS Institute, 1989, 1996). Phloem and sapwood were each analyzed separately for ethanol, water, and carbohydrate concentrations. Ethanol and water concentrations were analyzed as a split-plot design with treatment as the main plot and month as subplot. Soluble sugars and starch concentrations were analyzed separately for October and May as a one-way ANOVA with treatment as the main effect. Changes in concentrations of soluble sugars and starch between October and May were analyzed as a one-way ANOVA. Most May samples contained no starch and therefore were not analyzed statistically. α-Pinene concentrations were analyzed as a strip-plot with treatment as the main plot and tissue as a strip (Petersen, 1985). Data were tested for homogeneity of variance and normality before analysis and natural log-transformed when necessary. Back-transformed means and standard errors are presented for transformed data. Significant differences among means were identified by Fisher's protected LSD at $\alpha = 0.05$.

Regression analyses were performed separately for wet and crown logs to examine the relationships between rainfall and tissue water content, rainfall and tissue ethanol concentrations, water contents between tissues, ethanol concentrations between tissues, and the relationship between water content and ethanol

concentrations within tissues. Regression analysis also was performed for beetle numbers as a function of ethanol concentrations in May and August across all treatments. Phloem and sapwood ethanol concentrations were averaged for this analysis.

RESULTS

Ethanol concentrations in the phloem and sapwood were dependent only on the treatment, with no interaction between months (Figure 1A and B). The ethanol concentration in phloem from wet logs was 13.3 and 13.7 times higher than in dry or crown logs, respectively (both P values ≤ 0.001), with no difference between the latter two ($P = 0.972$). Similarly, the ethanol concentration in sapwood from wet logs was 31.3 times higher than in sapwood of dry logs ($P < 0.001$) and 6.9 times higher than sapwood of crown logs ($P = 0.021$). Sapwood of dry logs and crown logs had similar quantities of ethanol ($P = 0.084$).

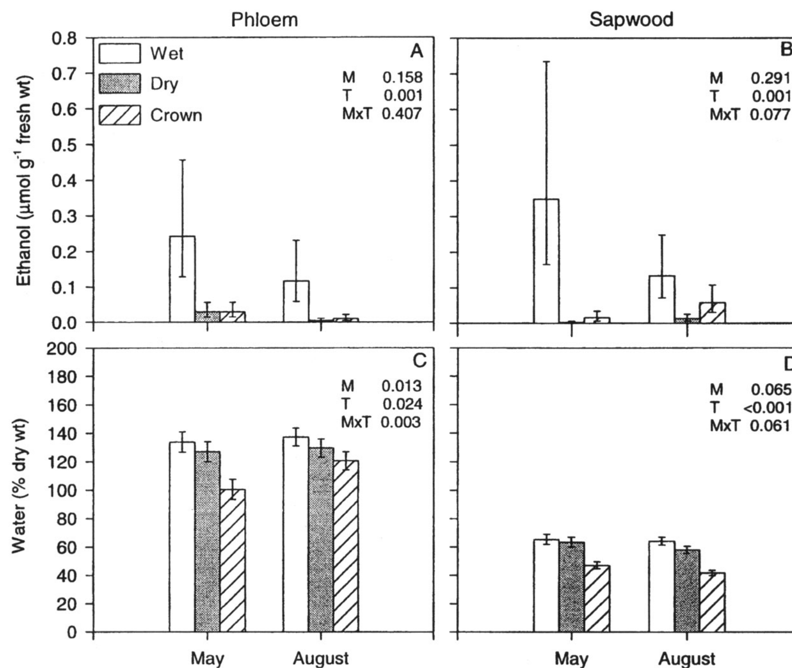

FIG. 1. Ethanol concentrations and water contents in the phloem and sapwood of wet, dry, and crown logs sampled in May and August 1994. Values are means ± SE. Insert lists P values for the ANOVA, M = month, T = treatment.

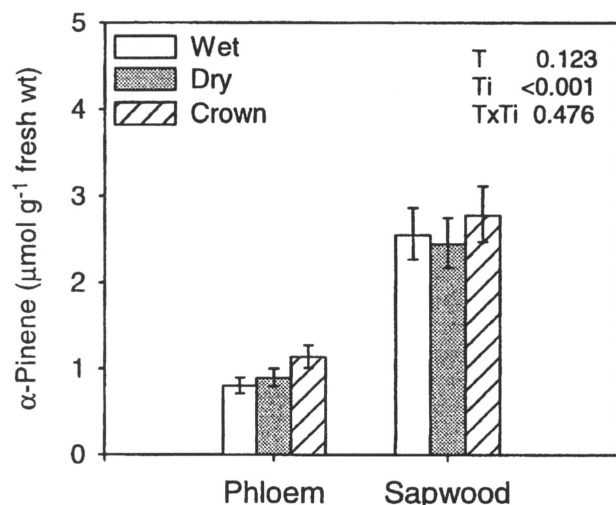

FIG. 2. α-Pinene concentrations in wet, dry, and crown logs sampled in May 1994. Values are means ± SE. Insert lists *P* values for the ANOVA, T = treatment, Ti = tissue.

α-Pinene concentrations in May were dependent only on the tissue type and not the treatment (Figure 2). Sapwood contained 2.8 times more α-pinene than the phloem ($P < 0.001$). Mean log diameters measured at mid-length were 45.7, 46.6, and 48.5 cm for dry, wet, and crown logs, respectively, with no significant difference among treatments ($P = 0.475$).

Rainfall through the canopy from November 8, 1993, to May 23, 1994, was 222.3 ± 18.3 cm (mean ± SE) near the wet logs. From May 24 to August 24, the rainfall was 17.2 ± 15.7 cm. Water content in the phloem was dependent on the treatment and time of year (Figure 1C), because of changes associated with crown logs. In May, the phloem of crown logs contained 24.8 and 20.9% less water than phloem of wet ($P = 0.002$) or dry logs ($P = 0.011$), respectively, with no difference between the latter two ($P = 0.491$). Water in the phloem of crown logs increased by about 20% between May and August ($P < 0.001$) with no changes occurring in the other treatments (both P values ≥0.400). Consequently, by August phloem water contents were not different among the three treatments (all P values ≥0.063). Sapwood water contents were dependent only on the treatment, with no interaction between months (Figure 1D). Sapwood from crown logs contained 68.4 and 73.1% of the water in sapwood from wet or dry logs, respectively (both P values <0.001). Wet and dry logs had similar sapwood water contents ($P = 0.252$).

Phloem and sapwood water contents from wet and crown logs were not related to the amount of rainfall. In crown logs, sapwood water content was

exponentially related to phloem water content ($y = e^{(3.27 + 0.0057x)}$; $r^2 = 0.56$, $P = 0.032$), but in wet logs there was no relationship. The ethanol concentration in phloem from wet logs was exponentially related to rainfall ($y = e^{-23.84 + 0.1009x}$; $r^2 = 0.79$, $P = 0.003$), and unrelated for crown logs. Sapwood ethanol concentrations were not related to rainfall in either treatment. The ethanol concentration in sapwood of wet logs was positively related to concentrations in the phloem ($y = 0.48 + 0.57x$; $r^2 = 0.98$, $P < 0.001$), while there was no relationship in crown logs.

Initial starch concentrations within tissues were similar for all treatments in October (Table 1). By the following May, the only starch remaining was in the sapwood of crown logs that retained about half the original amount. Changes in sapwood starch concentrations during the winter were greater in wet and dry logs compared to crown logs (both P values <0.001).

Total soluble sugar concentrations in October were much greater than starch in both the phloem and sapwood of all logs, with the highest quantities in the phloem (Table 1). The only difference for initial soluble sugar concentrations in phloem occurred between dry and crown logs ($P = 0.028$). In May, the soluble sugars remaining in the phloem of crown logs were 18 and 34% higher than in dry ($P = 0.027$) or wet ($P < 0.001$) logs, respectively, with no difference between the latter ($P = 0.063$). The change in soluble sugar concentration over winter was greater in the phloem of wet logs than in dry ($P = 0.009$) or crown logs ($P = 0.012$), with no difference between the dry and crown ($P = 0.904$). Sapwood of crown logs contained 26 and 32% more soluble sugars initially in October than either the wet or dry logs, respectively (both P values ≤ 0.006), with no difference between the latter two ($P = 0.570$). By May, the sapwood from crown logs contained 6.2 and 2.6 times more soluble sugars than the wet ($P < 0.001$) or dry ($P = 0.052$) logs, respectively, with no difference between the latter ($P = 0.067$). The change in sapwood soluble sugar concentration during winter was greater in wet logs than crown logs ($P = 0.033$), with no difference between wet and dry ($P = 0.490$) or dry and crown logs ($P = 0.138$).

Trypodendron lineatum and *Gnathotrichus retusus* were excavated and identified from pieces of logs on the study site. Although no *G. sulcatus* were excavated, our sample size was not large enough to assure their absence with confidence, and therefore we did not designate species within *Gnathotrichus*. *Trypodendron lineatum* galleries were found only in one wet treatment log and not analyzed statistically. In early September the density of *Gnathotrichus* spp. gallery entrance holes (Figure 3) in wet logs was 8.6 times higher than in dry logs ($P = 0.001$) and 3.7 time higher than in crown logs ($P = 0.011$), with no significant difference between the latter two treatments ($P = 0.252$). The densities of beetle galleries in September were significantly related to log ethanol concentrations in May (Figure 4), but not the concentrations in August.

TABLE 1. INITIAL (OCTOBER 1993), FINAL (MAY 1994), AND CHANGE IN CONCENTRATIONS (MEAN +SE–SE) OF STARCH AND TOTAL SOLUBLE SUGARS IN PHLOEM AND SAPWOOD OF DOUGLAS FIR LOGS SUBJECTED TO DIFFERENT TREATMENTS[a]

Tissue/ treatment	Starch (% dry wt)[b]			Total soluble sugars (% dry wt)		
	Initial	Final	Change	Initial	Final	Change
Phloem						
Wet	0.20 (+0.03–0.02)	0.00	−0.20 (+0.03–0.02)	8.36 (+0.26–0.25)	3.09 (+0.20–0.18)	−5.11 (+0.32–0.30)
Dry	0.18 (+0.02–0.02)	0.00	−0.18 (+0.02–0.02)	7.81 (+0.24–0.23)	3.53 (+0.22–0.21)	−4.25 (+0.27–0.25)
Crown	0.19 (+0.02–0.02)	0.00	−0.19 (+0.02–0.02)	8.61 (+0.27–0.26)	4.15 (+0.26–0.25)	−4.28 (+0.27–0.25)
P^c	0.693	—	0.693	0.077	0.001	0.013
Sapwood						
Wet	0.14 (+0.03–0.02)	0.00	−0.14 (+0.03–0.03)	1.08 (+0.06–0.06)	0.05 (+0.03–0.02)	−0.95 (+0.12–0.12)
Dry	0.15 (+0.03–0.02)	0.00	−0.15 (+0.03–0.03)	1.03 (+0.06–0.06)	0.12 (+0.07–0.04)	−0.85 (+0.12–0.11)
Crown	0.12 (+0.02–0.02)	0.07	−0.05 (+0.03–0.03)	1.36 (+0.06–0.06)	0.31 (+0.17–0.11)	−0.65 (+0.11–0.11)
P	0.489	—	0.000	0.003	0.002	0.090

[a]All means were back transformed, except for the changes in sapwood starch.
[b]Most samples had no starch in May, therefore statistical analysis was not done. Change = final − initial.
[c]P values for F tests for treatment differences from ANOVA.

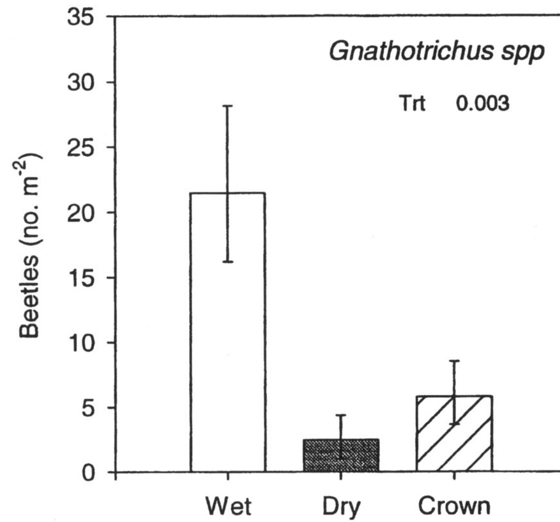

FIG. 3. Densities of *Gnathotrichus* spp. gallery entrance holes in the wet, dry, and crown logs sampled in September 1994. Values are means ± SE.

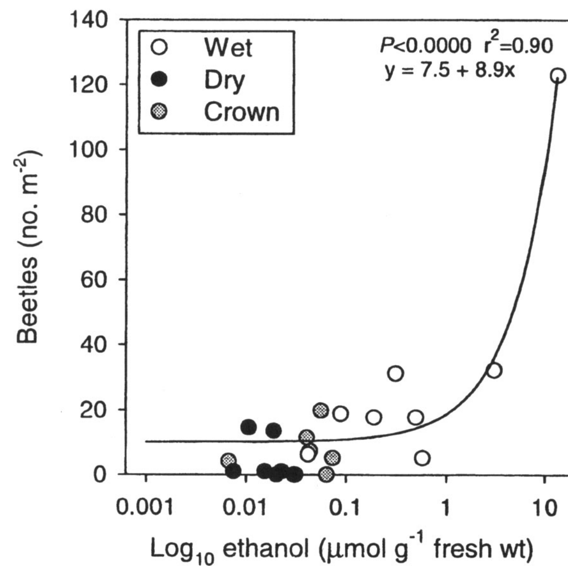

FIG. 4. The relationship between log ethanol concentrations (averaged for the phloem and sapwood) in May 1994 and the densities of *Gnathotrichus* spp. gallery entrance holes in September 1994.

DISCUSSION

Higher ethanol concentrations in wet logs than in dry logs demonstrates that rain contributes to the synthesis and accumulation of ethanol in coarse woody residues, which probably results from water interfering with gas exchange between respiring log tissues and the atmosphere. Tree tissues typically maintain constitutive levels of anaerobic enzymes (Kimmerer and Stringer, 1988; Harry and Kimmerer, 1991) that can quickly synthesize ethanol if O_2 becomes limited. For example, the phloem (with cambium) from Douglas-fir stems will produce high ethanol concentrations (58–79 μmol/g dry wt) in 4 hr when sealed in a vial with an anoxic N_2 atmosphere (Kelsey et al., 1998). The phloem will also produce ethanol (6–11 μmol/g dry wt) within 4 hr if the vial contains air, because aerobic respiration reduces the O_2 concentration low enough to initiate ethanol synthesis. Similarly, ethanol synthesis is initiated in the cambium of eastern cottonwood (*Populus deltoides* Bartr.) when whole logs are exposed to low oxygen levels (3–7%) for 6 hr (MacDonald and Kimmerer, 1991). Consequently, we suspect that rain on logs creates a barrier to gas exchange with the atmosphere, and the O_2 concentrations decrease as aerobic respiration proceeds in the tissues. The more frequently that rain interferes with the gas exchange, or the longer that a water barrier remains, the higher the ethanol concentrations produced.

While ethanol synthesis occurred in the phloem, cambium, and sapwood of wet logs, it appears that the water barrier occurred in the outer bark and not in these underlying tissues, for two reasons. First, there was no relationship between rainfall and water contents in the phloem or sapwood, yet there was a relationship between rainfall and ethanol concentration in the phloem. Second, the phloem and sapwood from wet logs produced substantially higher ethanol concentrations than in dry logs, but their tissues had similar water contents in May. This suggests that the phloem and sapwood in wet and dry logs had the same water contents throughout the winter months because both log treatments were exposed to the same atmospheric vapor pressure deficits. These observations are consistent with water filling the air spaces in outer bark and creating a barrier to gas exchange. Since outer bark water content can fluctuate rapidly depending on rainfall patterns and evaporation (Harmon and Sexton, 1995), the formation and existence of a water barrier is probably a dynamic process that could contribute, in part, to the large variation in ethanol concentrations observed among logs subjected to similar environmental conditions in this and in other work (Kelsey, 1994a,b).

Crown logs were exposed to the same winter rainfall as wet logs, but in May they were drier and contained much lower ethanol concentrations than wet logs, as observed in other work (Kelsey 1994b). Water was apparently being transported up the stem and evaporating through the needles. This movement probably caused phloem water to move more rapidly into the sapwood, because there was a relationship between phloem and sapwood water contents in crown logs,

but not wet logs. Between May and August the phloem water content of crown logs increased with summer rains, probably because water movement stopped when the needles dried.

Water movement in crown logs might affect ethanol accumulation in several ways. It could disrupt the formation of a water barrier or reduce its functional longevity, thus allowing the tissues to remain aerobic and synthesize less ethanol. If rain absorbed in crown logs moves quickly through the outer bark into the phloem and then up the sapwood into the crown, the water may carry some dissolved oxygen into the tissues and help minimize hypoxia. Increased tension in the sapwood water column, as a result of continued evaporation loss from the crown, also can cause the water columns to cavitate and the tracheids to fill with air (Tyree and Sperry, 1989), which will contain oxygen if it enters from the cut ends of logs. This could improve the gas exchange in sapwood adjacent to the cambium and phloem, thus minimizing ethanol synthesis. Alternatively, the tissues of crown logs may have produced the same quantity of ethanol as wet logs, but the ethanol was transported with water up the stem and into the crown where it was metabolized in needles or other tissues that are more aerobic (Jayasekera et al., 1990; MacDonald and Kimmerer, 1993).

Ethanol concentrations in Douglas fir from the McDonald Forest were very low compared to those in logs at a site near the Columbia River (118.5 and 111.8 μmol/g fresh wt in phloem and sapwood, respectively) about 175 km north (Kelsey and Joseph, 1997). Such large differences in ethanol concentrations between sites may be caused primarily by rainfall and solar radiation. McDonald Forest is normally drier than the area near the Columbia river. In addition, the logs in McDonald Forest were located on the northwest side of a ridge beneath a canopy with 78% closure. In addition to intercepting rainfall, the canopy shaded the logs and kept them cooler. In contrast, logs near the river were located on a ridge, at the top of a clear-cut with no cover and maximum exposure to rainfall and solar radiation. The amount of solar radiation is important because it affects tissue temperatures and metabolic rates (Sprugel et al., 1995). As temperature and aerobic respiration rates increase, tissue O_2 level may be depleted faster if gas exchange with the atmosphere is impaired. Furthermore, once ethanol synthesis begins, the rate will also increase as temperatures rise (authors' unpublished data). The combination of high solar radiation in conjunction with a water barrier to gas exchange probably represents the optimum condition for maximizing ethanol synthesis and accumulation in logs.

The effectiveness of leaving branches or crowns on logs as a means of mitigating ambrosia beetle gallery densities will probably vary with the environmental conditions. We have not examined ethanol accumulation in branched logs with full exposure to rain and solar radiation. Under these conditions they would probably produce more ethanol than measured here, thus making them susceptible to attack by a greater number of ambrosia beetles.

The importance of ethanol as a host attractant for the *Gnathotrichus* spp. is evident from their selection of logs with higher ethanol concentrations. This response is consistent with previous observations with logs (Kelsey, 1994a,b; Kelsey and Joseph, 1997) and the observations of others, who used chemical baits (Liu and McLean, 1989; Salom and McLean, 1990). Although the logs contained very low ethanol concentrations, they were still susceptible to some attack by *Gnathotrichus* spp., and the beetles retained their ability to discriminate between different ethanol concentrations. α-Pinene by itself did not contribute to the beetles' selection because all logs had the same α-pinene concentrations in May. In addition, western hemlock and Douglas fir logs have been observed with similar densities of *Gnathotrichus* spp. galleries, but the western hemlock tissues contained minimal quantities of α-pinene compared to Douglas fir (Kelsey and Joseph, 1997). Using baited traps, Liu and McLean (1989) concluded that α-pinene was not a primary host attractant or a synergist for the aggregation pheromones of *G. sulcatus* or *G. retusus*. Our results support this conclusion.

The densities of ambrosia beetle galleries in logs from this experiment were low compared to our other studies (Kelsey 1994a; Kelsey and Joseph, 1997), probably in part because the surrounding beetle populations were low as a consequence of limited host material for them to colonize in the adjacent forest prior to the experiment. Furthermore, our study site represented only a small portion of the total area thinned, so there was abundant host material for the existing beetles to colonize, thus diluting the gallery densities. Ethanol concentrations in the logs also may not have been high enough to attract greater numbers of beetles.

Carbohydrates are the substrate for both aerobic and anaerobic respiration. Prior to the detection of ethanol in aging logs (Moeck, 1970; Cade et al., 1970), ambrosia beetles were shown to be attracted to logs with a low starch or no starch content, but not all logs without starch attracted beetles (Chapman et al., 1963; Chapman and Dyer, 1969). Our results provide an explanation for these early observations. If the starch and soluble sugars in log tissues are depleted primarily by aerobic respiration, as in dry logs, then ethanol synthesis would be minimal and ambrosia beetles will not be attracted. If a significant portion of the starch and soluble sugars are metabolized by anaerobic respiration, as in wet logs, then ethanol will accumulate and attract beetles. Crown logs in May retained more starch in the sapwood and more soluble sugars in the phloem and sapwood than wet or dry logs, suggesting that needles on the crown may have contributed some photosynthate and reduced the net depletion of carbohydrates in log tissues.

In conclusion, rain strongly influences the accumulation of ethanol in aging log tissues, probably by creating a barrier to gas exchange between respiring log tissues and the atmosphere. Ethanol concentrations can be minimized by keeping the logs dry, or leaving their branches attached, but the effectiveness of the

latter may vary depending on environmental conditions. Although log ethanol concentrations were relatively low compared to another experiment (Kelsey and Joseph, 1997), *Gnathotrichus* spp. ambrosia beetles were still able to discriminate between logs containing different quantities of ethanol, with a preference for high concentrations. Gallery densities of *Gnathotrichus* spp. can be minimized by limiting ethanol synthesis and accumulation in logs tissues. Manipulating ethanol synthesis in logs and coarse woody materials may be useful in developing management alternatives for controlling undesirable behavior of ambrosia beetles and other forest insects attracted to ethanol.

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CHEMICAL DEFENSE OF THE CARIBBEAN REEF SPONGE *Axinella corrugata* AGAINST PREDATORY FISHES

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Abstract—Field and laboratory experiments were performed to investigate the palatability to predatory fishes of organic extracts and purified compounds from the Caribbean reef sponge *Axinella corrugata* (=*Teichaxinella morchella*). When incorporated into artificial foods at the same volumetric concentration as found in sponge tissue, crude extracts of the sponge, as well as a butanol-soluble partition of the crude extract, deterred feeding of the Caribbean reef fish *Thalassoma bifasciatum* in laboratory aquarium assays and deterred feeding of a natural assemblage of fishes in assays performed on reefs where *A. corrugata* is found. Bioassay-directed fractionation of the butanol-soluble partition led to the isolation of a single compound responsible for feeding deterrency, stevensine, a previously described dibrominated alkaloid. The mean concentration of stevensine in *A. corrugata*, as determined by quantitative NMR analysis, was 19.0 mg/ml (N = 8, SD = 7.2 mg/ml). Stevensine deterred feeding in laboratory aquarium assays at concentrations >2.25 mg/ml, and deterred feeding in field assays at ~12 mg/ml. Stevensine represents another in the oroidin class of brominated pyrrole derivatives that function as chemical defenses of sponges in the families Axinellidae and Agelasidae.

Key Words—Chemical defense, sponges, predation, Caribbean, *Teichaxinella, Axinella, Agelas*, brominated metabolites.

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INTRODUCTION

Marine invertebrates that lack physical protection may use alternative defensive strategies such as chemical defense. Many of the unusual secondary metabolites that have been isolated from soft-bodied marine invertebrates are proposed to function as agents that deter predation (reviews in Paul, 1992a; Pawlik, 1993). Only recently have ecologically relevant field and laboratory experiments been designed to test this hypothesis, and our knowledge of the ecological roles of invertebrate secondary metabolites has increased rapidly over the last decade (e.g., Pawlik et al., 1987; Harvell et al., 1988; Becerro et al. 1994; McClintock et al., 1994; Pennings et al., 1994; Hay, 1996).

Sponges are the single most diverse source of marine natural products (Braekman et al., 1989; reviewed in Faulkner, 1998, and previous reviews cited therein); these compounds have exhibited potentially important activity in pharmacological studies (e.g., Kitagawa and Kobayashi, 1993; Munro et al., 1994), but their ecological functions remain to be determined. Compounds isolated from sponges vary widely in structural complexity; compound classes include sterols, terpenoids, amino acid derivatives, saponins, and macrolides (Sarma et al., 1993; Faulkner, 1998). Many of these compounds have complex carbon skeletons that are nitrogen- or halogen-rich (Faulkner, 1998; Paul, 1992a). Concentrations of secondary metabolites in sponge tissues can be quite high; for example, scalaradial constituted 2.4% of the total dry mass in the Pacific sponge *Hyrtios erecta* (Rogers and Paul, 1991).

The structural complexity and high concentration of the secondary metabolites isolated from sponges suggest they play an important ecological function (Paul, 1992a; Pawlik, 1993). Among the proposed roles, sponge secondary metabolites have been implicated in sponge–coral allelopathic interactions (Sullivan and Faulkner, 1983; Porter and Targett, 1988), the inhibition of settlement of larval fouling organisms (Davis et al., 1991; Pawlik, 1992; Henrikson and Pawlik, 1995, 1998), and in the protection of sponges from microorganisms and ultraviolet radiation (Paul, 1992a). The most prevalent theory regarding the function of sponge secondary metabolites is that they act to deter potential predators (e.g., Paul, 1992b; Pawlik, 1993). Sponges are soft-bodied and sessile, and thus appear to be physically vulnerable to predation. Caribbean demosponges, for example, represent a rich protein source [mean of 20.7 mg soluble protein per milliliter of tissue, $N = 71$ (Chanas and Pawlik, 1995)] in an environment noted for intense grazing activity by fishes (Hixon, 1983; Jones et al., 1991). Nevertheless, very few fish species are known to feed on Caribbean sponges (Randall and Hartman, 1968). Only recently have ecologically relevant methods been used to test whether sponge secondary metabolites deter consumption by predatory fishes (Thompson et al., 1985; Pawlik et al., 1988; Herb et al., 1990; Rogers and Paul, 1991; Duffy and Paul, 1992; Albrizio et al., 1995).

In our recent survey of the chemical antipredatory defenses of 73 species of Caribbean sponges (Pawlik et al., 1995), we discovered that all of the five species within the family Axinellidae yielded crude organic extracts that deterred the feeding of predatory reef fish in aquarium assays. One species, *Axinella corrugata* (previously *Teichaxinella morchella*) consistently yielded a highly deterrent crude extract (0 of 10 food pellets eaten). The purpose of the study reported herein was to isolate and identify the metabolite(s) responsible for the chemical defense of *A. corrugata* using bioassay-guided fractionation techniques.

METHODS AND MATERIALS

Sponge Collection. Samples of *Axinella corrugata* used for assays were collected in June and July 1996 on shallow (depth <25 m) reefs surrounding the Bahamas Islands while on board the *R/V Seward Johnson.* Additional samples obtained for analysis of intraspecific variability of metabolite concentration were collected from reefs off Key Largo, Florida, in July and August 1996. Individual sponges were collected by cutting the narrow base of the sponge from the substratum with a sharp knife. Sponges were extracted immediately or stored at −20°C until used.

Extraction and Isolation. For preliminary field and aquarium assays, sponge samples were processed immediately after collection, while later analyses used frozen material. Fresh or frozen sponge was cut into small pieces and placed in a 1000-ml graduated cylinder containing 500 ml of 1 : 1 dichloromethane–methanol (DCM/MeOH). Sponge tissue was added until the displaced volume reached 1000 ml. The tissue was thoroughly extracted twice in 1 : 1 DCM/MeOH and once in MeOH, each for >12 hr. The resulting extracts were combined and divided into aliquots, each from a known volume of sponge tissue. Some of these aliquots were used for assays of the crude extract or for later quantification, while the majority were used for bioassay-directed fractionation.

Aliquots of crude extract representing a known volume of sponge were combined and successively partitioned with organic solvents of increasing polarity. The crude extract was initially partitioned between water and 2,2,4-trimethylpentane (TMP), then between water and DCM, and finally between water and butanol (~500 ml of each solvent), leaving four solvent partitions: TMP, DCM, butanol, and aqueous. Each partition was filtered through Celite and dried by rotary evaporation before being subjected to feeding assays and further separation.

Using aquarium assays, fish feeding deterrent activity was largely isolated in the butanol partition. Partitions were analyzed using C_{18}-reverse phase thin-layer chromatography (TLC; methanol elution, UV visualization). Subsequent

purification of constituent compounds from the butanol partition employed size-exclusion chromatography using lipophilic Sephadex LH-20, with methanol elution. Resulting fractions were analyzed using TLC, nuclear magnetic resonance (NMR) spectrometry, high-resolution mass spectrometry (HR-MS), and fractions were subjected to aquarium assays. Comparison of the NMR and HR-MS data with the literature enabled structural assignment of the active metabolite.

To determine the concentration of the active metabolite in butanol partitions of crude extracts from sponge samples from different locations, quantitative proton NMR analysis was used. Integration of proton signals arising from both pure samples of the active compound and a known amount of an internal standard (cyclooctatetraene) provided an estimate of the mass of the active compound in the butanol partition from a known volume of sponge tissue.

Laboratory Feeding Assays. Aquarium assays were performed as described in Pawlik et al. (1995) on the crude extract, each solvent partition, each fraction, and the purified active metabolite from *Axinella corrugata*. Assays were performed on board the research vessel *Seward Johnson* or in aquaria at the University of North Carolina at Wilmington by employing a common predatory reef fish, the bluehead wrasse *Thalassoma bifasciatum*. The advantages of using this species for aquarium bioassays have previously been detailed (Pawlik et al., 1987, 1995). Groups of three fish (one terminal phase male, two females) were held in each of 15–20 separate, opaque-sided compartments in flow-through laboratory aquaria. Groups of 10 fish were chosen out of 15 at random during feeding assays and randomly offered either a treated or control food pellet, followed by the other choice. When the second pellet was a treated pellet and was rejected, a third pellet was offered as a control to determine whether the fish had ceased feeding. Groups of fish that did not eat control pellets were considered satiated and were not used in the experiment. A food pellet was considered rejected if one or more fish took the pellet into their mouths and expelled it at least three times or if the pellet was approached and ignored. The significance of differences in the consumption of treated versus control pellets was evaluated with the Fisher exact test (Zar, 1984). For any single assay of 10 replicates, an extract was significantly deterrent if four or more of the pellets were rejected ($P \geq 0.043$, one-tailed test); therefore, a sample was considered deterrent if the number of pellets eaten was less than or equal to 6.

Food pellets for laboratory assays were made by mixing a volume of 5 g of lyophilized, macerated squid with 3 g of alginic acid and 100 ml of water, followed by addition of the volumetric equivalent of a fraction or compound in a carrier solvent (for treated pellets) or solvent alone (for control pellets). The mixture was vigorously stirred to remove lumps and then loaded into a 5-ml syringe. The tip of the syringe was then dipped into a beaker filled with a 0.25 M solution of $CaCl_2$ and the contents of the syringe slowly expelled to form a long strand. After a few minutes, the strand was removed, rinsed in seawater,

and chopped into 4-mm-long pieces with a razor blade to form uniform pellets.

Field Feeding Assays. Feeding experiments were conducted in the field on each of three samples: the crude extract, the butanol partition, and the purified active compound. In each case, the sample was volumetrically reconstituted in a matrix of carrageenan at the concentrations as they occurred in the sponge. Strips of this matrix were then used in field experiments (see methods in Fenical and Pawlik, 1991). The matrix was made by combining 1.5 g of carrageenan (Gelcarin, FF961L; FMC Corp, Philadelphia, Pennsylvania) and 3 g of lyophilized, macerated squid with deionized water to a total volume of 65 ml. The mixture was heated to boiling in a microwave oven (about one minute on "cook"), then the sample was added with a minimal volume of solvent (treated matrix) or solvent alone (control matrix). Food dye was added to the control matrix so as to color-match the treated matrix. The mixture was stirred and heated again to boiling (pure compounds were later judged to be stable to this treatment). The molten mixture was then poured into plastic molds crossed by lengths of cotton string that protruded from the ends of the molds. After the matrix had cooled and set, 1.0- × 0.5- × 5.0-cm strips were sliced to size with a razor blade and removed from the mold. For each experiment, 20 treated strips and 20 control strips were prepared. To distinguish treated from control strips, the cotton string attached to each strip was marked with a small ink spot.

Field assay methods are described in detail in Pawlik and Fenical (1989, 1992). One treatment and one control strip each were tied to a 50-cm length of three-strand nylon rope at a distance of approximately 4 and 12 cm from one end of the rope (the order was haphazard). Twenty ropes were deployed on the same reefs from which *Axinella corrugata* had been collected, with the end of each rope opposite the food strips attached to the substratum by inserting a piece of coral or rock through the rope twines. Within 1 hr, the ropes were retrieved and the amount of each strip eaten was recorded as a percentage decrease in the strip length (to the nearest 5%). The Wilcoxon paired-sample test (one-tailed) (Zar, 1984) was employed to analyze the results after excluding pairs for which both control and treatment slices had been either completely eaten or not eaten at all.

RESULTS

Assay food strips containing a crude extract of *Axinella corrugata* at the same volumetric concentration as the extract occurs in the tissues of the sponge significantly deterred feeding of a natural assemblage of consumers present on the reef ($P < 0.001$, Wilcoxon paired-sample test; Figure 1). For this and for subsequent field feeding assays, food strips were observed being consumed by

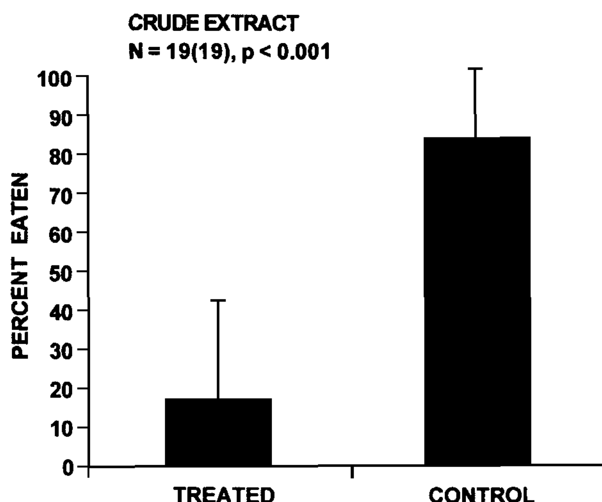


FIG. 1. Field assay. Feeding by reef fishes on paired control food strips and strips containing a crude extract of *Axinella corrugata* at the same concentration as found in the tissues of the sponge; 1 SD above the mean is indicated; N = number of paired treatment and control strips retrieved of the 20 deployed (number of pairs used in statistical analysis). Probability calculated using the Wilcoxon paired-sample test.

a wide variety of reef fishes, particularly wrasses (*Thalassoma* and *Halichoeres* spp.), snappers (*Ocyurus chrysurus*), parrotfishes (*Scarus* and *Sparisoma* spp.), grunts (*Haemulon* spp.), tilefish (*Malancanthus plumieri*), porgy (*Calamus* spp.) and angelfishes (*Pomacanthus* and *Holacanthus* spp.).

In laboratory aquarium assays employing the reef fish, *Thalassoma bifasciatum*, the crude extract of *Axinella corrugata* and the butanol partition of the crude extract significantly deterred feeding (<2 pellets eaten of 10 offered). Weak feeding deterrent activity was observed in the aqueous partition. The butanol partition also significantly deterred feeding of reef fishes in a field assay ($P < 0.005$; Figure 2).

Bioassay-guided fractionation of the butanol partition of the crude extract using LH-20 Sephadex chromatography resulted in the isolation of active fractions containing a single metabolite. This metabolite was identified as stevensine (Figure 3) by NMR spectroscopy and high-resolution mass spectroscopy and comparison with data reported in the literature for both naturally occurring and synthetically produced stevensine (Albizati and Faulkner, 1985; Wright et al., 1991; Xu et al., 1997).

Volumetric concentrations of stevensine in the tissues of individual specimens of *Axinella corrugata* from eight locations in the Bahamas and Florida

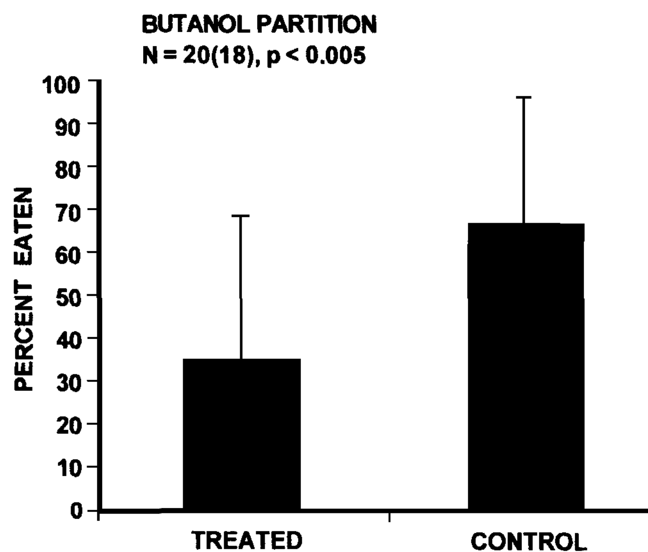
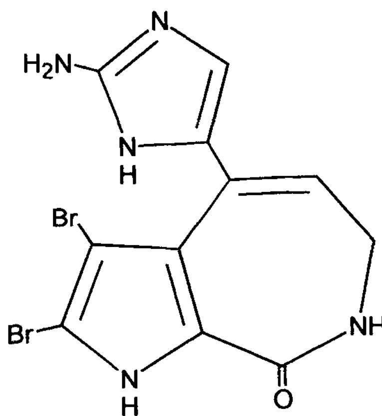


FIG. 2. Field assay. Feeding by reef fishes on paired control food strips and strips containing a butanol partition of the crude extract of *Axinella corrugata*. Details as in Figure 1.



STEVENSINE

FIG. 3. The structure of stevensine, defensive metabolite present in the tissues of *Axinella corrugata*.

TABLE 1. VOLUMETRIC CONCENTRATION OF STEVENSINE IN TISSUES OF INDIVIDUAL SPECIMENS OF *Axinella corrugata* COLLECTED IN BAHAMAS ISLANDS AND FLORIDA AS DETERMINED BY QUANTITATIVE NMR ANALYSIS

Location	Date (in 1996)	Depth (m)	Concentration (mg/ml tissue)
Bahamas Islands			
Sweetings Cay	Jun 18	22	12.0
Sweetings Cay	Jun 19	15	17.0
Little San Salvador Is., reef	Jun 25	22	30.0
Little San Salvador Is., pinnacles	Jun 25	22	27.0
Andros Island	Jul 2	18	25.0
Chubb Cay	Jul 1	28	12.0
Key Largo, Florida Keys			
Conch Reef	Aug 2	15	13.0
Molasses Reef	Jul 31	15	16.0
Mean concentration			19.0 ± 7.2

Keys were determined using quantitative proton NMR (Table 1). Four proton signals from stevensine (δ 6.23, 6.85, 7.39, 8.15 ppm) were integrated, and the mean was compared with the proton signal derived from the internal standard (COT; δ 5.7 ppm). Stevensine concentrations ranged from 12 to 30 mg/ml sponge tissue (mean = 19.0 ± 7.2 mg/ml).

Purified stevensine deterred feeding of a natural assemblage of reef fishes in a field assay in which the compound was assayed at the low end of the concentration range exhibited in the tissues of *Axinella corrugata* (~12 mg/ml; $P < 0.05$; Figure 4). Stevensine was also subjected to a series of laboratory assays at decreasing concentrations (Figure 5). The minimally active concentration at which stevensine deterred feeding of the wrasse *Thalassoma bifasciatum* was 2.0–2.25 mg/ml.

DISCUSSION

This study is part of a broader program that developed from a recent survey of the crude extracts of 73 species of Caribbean sponges for antipredatory chemical defenses (Pawlik et al., 1995). Deterrent metabolites appear to be the principal defensive strategy of Caribbean sponges against predatory reef fishes because no evidence has been found for structural or nutritional defenses in a similarly broad survey (Chanas and Pawlik, 1995, 1996). Identification of the metabolites responsible for sponge chemical defenses will provide a better understanding of structural components required for distastefulness, as well as the evolution of chemical defenses. To date, a new polymeric pyridinium alkaloid, amphitoxin,

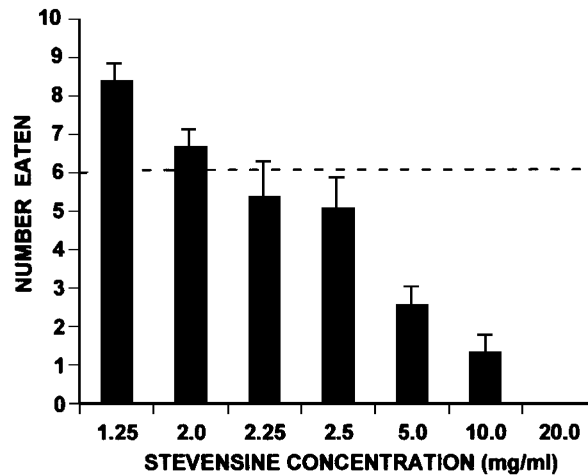


FIG. 4. Aquarium assay. Consumption by *Thalassoma bifasciatum* of control food pellets and pellets (mean + SE) containing a range of concentrations of stevensine. Fish consumed all 10 control pellets in all cases. Three replicate assays were performed at each concentration. For any individual assay, the treatment was considered deterrent if the number of pellets eaten was less than or equal to 6 ($P < 0.04$, Fisher exact test, one-tailed), as indicated by the dotted line on the graph.

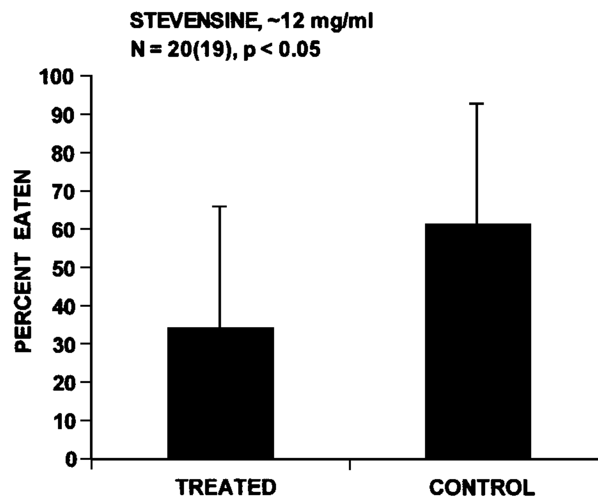


FIG. 5. Field assay. Feeding by reef fishes on paired control food strips and strips containing purified stevensine from *Axinella corrugata* at ~12 mg/ml. Details as in Figure 1.

has been isolated as the chemical defense of *Amphimedon compressa* (Albrizio et al., 1995), and oroidin and its carboxypyrrole hydrolysis product have been identified as the primary defensive agents of Caribbean sponges of the genus *Agelas* (Cahans et al., 1996).

Brominated pyrrole derivatives are common secondary metabolites in sponges of the families Axinellidae and Agelasidae, with *Axinella corrugata* a member of the former. These two families were once included in the order Axinellida, partially on the basis of similar secondary metabolite chemistry (Bergquist, 1978). The two families have since been separated at the ordinal level due to marked differences in skeletal structure (Hartman, 1982), and the family Axinellidae has been allocated to different orders and the genera reassigned based on morphological characteristics (Alvarez and Crisp, 1995; Alvarez et al., 1998). Brominated pyrroles have exhibited a wide range of biological effects in pharmacological assays, including antiviral and antibacterial activity (e.g., Keifer et al., 1991) and have been used as chemotaxonomic indicators (Braekman et al., 1992). Stevensine is closely related to the compound oroidin, which is commonly extracted from the tissues of sponges of the genus *Agelas* (Forenza et al., 1971; Braekman et al., 1989, 1992). Oroidin from Caribbean species of *Agelas* has been demonstrated to act as a chemical defense against fish predators (Chanas et al., 1996). Whether these compounds also serve as chemical defenses against potential invertebrate predators, or function as antimicrobial agents or as inhibitors of overgrowth or fouling, remains to be investigated.

The majority of sponges on Caribbean reefs elaborate secondary metabolites that serve as defenses against predatory reef fishes (Pawlik et al., 1995). Discovery of the compounds responsible for chemical defenses has only just begun. Further isolation and identification will allow for comparisons of the deterrent chemistry to reveal structure–function and chemotaxonomic relationships. Also interesting is the metabolic cost of these chemical defenses and its impact on sponge growth and fecundity, particularly because not all reef sponges employ deterrent meabolites, and these chemically undefended species are often eaten by spongivorous fishes (Pawlik et al., 1995; Pawlik, 1998). In addition, it is likely that many identified sponge natural products will not play a role in deterring predatory reef fishes. Other defensive functions of secondary metabolites (or additional functions of identified deterrent compounds) may include deterring invertebrate predators, antifouling, anti-overgrowth, or protection from UV radiation (Paul, 1992a; Pawlik, 1993). Stevensine has recently been shown to have weak antimicrobial activity against a test panel of marine bacteria that included strains isolated from necrotic sponges (Newbold et al., 1999), providing evidence that some secondary metabolites may serve multiple defensive roles.

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QUANTITATIVE STRUCTURE–ACTIVITY RELATIONSHIPS (QSAR) OF CINNAMIC ACID BIRD REPELLENTS

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Abstract—Plants have evolved an array of defense chemicals that inhibit the feeding of vertebrate herbivores and therefore have potential for agricultural and environmental applications to reduce feeding damage. We investigated the relationship between structure and repellency for 14 derivatives of the plant secondary compound, cinnamic acid, using the feral pigeon (*Columba livia*) as the test species. The mechanism behind the repellent activity of these derivatives is explained by a combination of four descriptors: heat of formation (ΔH_f), polarizability (XY and YY) and superdelocalizability (Sr). All these parameters are electronic, indicating that changes in electronic distribution within cinnamic acid structures are crucial for activity. This is the first published quantitative structure–activity model for avian repellents, and as a result we can now begin to predict which cinnamic acid derivatives should make effective repellents. The full power of this model to aid the selection and screening of new repellents awaits further experimentation on both related compounds and other avian species. However, this modeling approach promises to provide a more efficient and economic method for prospecting chemical databases for new effective bird repellents.

Key Words—Cinnamic acids, bird repellents, quantitative structure–activity relationship, dose–response, pigeon, *Columba livia*, crop protection, feeding deterrent.

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INTRODUCTION

The use of chemical repellents has considerable potential as a relatively benign method of reducing the damage caused by pest species. However, few effective repellents are currently available for vertebrate pests. This is perhaps due to the largely empirical strategies used for the selection of chemicals as repellents. Screening programs were often targeted at compounds already registered for other agricultural or pharmaceutical applications (Woronecki et al., 1981; Brooks and Rowe, 1987; Avery and Decker, 1991). In practice, the effect of many such chemicals was found to be short-lived, or the compound was toxic and therefore not widely used.

More recently research has focused on identifying compounds that are biologically significant to the particular pest we wish to repel (Watkins et al., 1996a). Secondary plant substances (e.g., phenylpropanoids, phenols, and terpenoids) have a profound effect on food-plant selection by many herbivores. The presence of such chemicals can serve as a warning that the plant is unpalatable and encourages herbivores to feed on other plant species. The response to these compounds is mediated by the chemical senses (i.e., taste, olfaction, and trigeminal chemoreception) and/or postingestional effects (i.e., gastrointestinal malaise). The aversive response to some chemical repellents is innate, a property that is the result of past evolutionary pressures to develop sensitivity to particular odors or tastes. Foods that are toxic often taste bitter or cause irritation to the buccal cavity, although this relationship is by no means perfect. For example, mammals show aversive orofacial responses to quinine and capsaicin (the pungent principle of chili peppers) despite having no prior experiences of these compounds (Chambers and Bernstein, 1995). Experience can also play a critical role in the response to a repellent. An initial preference for treated food is reversed when the post-ingestional consequences of eating the food are negative. The compound causes some form of transient upper gastrointestinal discomfort or illness such as nausea or vomiting, which the individual then associates with the taste of the compound or, if the compound has no taste, another salient cue within the food (Provenza, 1995). The animal then becomes conditioned to avoid that cue in future encounters. In agriculture, this latter type of repellency has been successfully used to train livestock to avoid certain plant species (Burrit and Provenza, 1990).

These compounds, because of their "evolved" defensive function, are well suited for agricultural and environmental applications to reduce feeding damage (Mason and Clark, 1992; Jakubas et al., 1992). A small number are already undergoing commercial evaluation in applications to prevent bird damage to crops (Cummings et al., 1995; Gill et al., 1994, 1998), inhibit nontarget wildlife from consuming potentially toxic granular pesticides and chemically treated

seeds (Mason et al., 1993; Watkins et al., 1996b), and prevent gnawing damage to electrical cables by rodents (Kurata et al., 1994).

To date the discovery of chemical repellents has been more by chance than by design. Such chance discovery has limited the number of economically viable and environmentally safe repellents. Each step in the development of a useful repellent is a sifting process with technical, safety, and economic factors all playing a role in determining the fate of any candidate compound (Mason and Clark, 1992). Thus, if the initial number of candidate compounds is few, the likelihood of any compound passing through each of the above sieves is also likely to be small. In order to boost the number of candidate compounds quickly and efficiently, we and other research groups have been seeking to generate rule-based systems by which we can select or reject a compound as a repellent based simply on an examination of its chemical structure.

Qualitative models have recently been developed that can predict the repellent activity of aromatic, plant-derived compounds for both birds and mammals (Clark and Shah, 1994; Nolte et al., 1993). For avian repellents, these models (Shah et al., 1991; Jakubas et al., 1992; Clark and Shah, 1994) predict that the most critical features, at least for certain classes of compound (e.g., anthranilates, acetophenones, benzoates, vanilates, and cinnamic acid derivatives) are the presence of an electron-rich phenyl ring with electron withdrawing and donating groups that are held within a resonant substitution pattern (Clark and Shah, 1994).

The current study set out to build on this previous work and generate a quantitative model with which to describe the relationship between the structure and the activity of bird repellents. It focused on a series of cinnamic acid derivatives for which we had evidence of repellency against birds, mammals, and invertebrates (Crocker and Reid, 1993; Gurney et al., 1996; Mosson et al., 1996). Metabolites of cinnamic acid, isolated from the flower buds of a bird-resistant variety of pear tree, have been found to deter birds from feeding (Crocker and Perry, 1990). When these compounds and their derivatives were presented individually to captive feral pigeons (*Columba livia* Gmelin) in laboratory no-choice feeding trials, several proved to be effective feeding deterrents (Crocker et al., 1993; Watkins et al., 1995) that could offer a large margin of safety in terms of their low toxicity (Hoskins, 1984). These studies also showed that despite the superficial similarity of their structures, the cinnamic acid derivatives differed greatly in their repellency. For example, birds avoided food treated with 3,5-dimethoxycinnamic acid and yet were indifferent to 3,4-dimethoxycinnamic acid (Crocker and Perry, 1990). A simple explanation of these differences, however, was not apparent. This paper reports on a subsequent study whose aim was to establish the dose–response relationships of these compounds and, using computer-aided software, to explore the relationship between their efficacy and key topological and electronic parameters.

METHODS AND MATERIALS

Chemicals. The *trans* isomers of 2-methoxycinnamic acid (C.A.S. 6099-03-2), 3-methoxycinnamic acid (C.A.S. 6099-04-3), 4-methoxycinnamic acid (C.A.S. 830-09-1), 3,5-dimethoxycinnamic acid (C.A.S. 1132-21-4), 3,4-hydroxycinnamic acid (C.A.S. 331-39-5), 3-methoxy-4-hydroxycinnamic acid (C.A.S. 537-73-5), 3,5-dimethoxy-4-hydroxycinnamic acid (C.A.S. 530-59-6), 3-chlorocinnamic acid (C.A.S. 1866-38-2), 3-nitrocinnamic acid (C.A.S. 555-68-0), cinnamic acid (C.A.S. 140-10-3), cinnamamide (C.A.S. 621-78-1), cinnamyl alcohol (104-54-1), cinnamaldehyde (C.A.S. 14371-10-9), and 3,5-dimethoxybenzoic acid (C.A.S. 1132-21-4) were supplied by Aldrich Chemical Company (Gillingham, Dorset, UK). Solvents (HPLC grade) were supplied by Rathburn (Walkerburn, Peebleshire, UK) and potassium dihydrogen orthophosphate by Merck Chemical Company (Lutterworth, Leicester, UK).

Sample Preparation. Chicken layer pellets (Lillico & Son, Reigate, Surrey, UK) were used as the coating substrate. These pellets are readily accepted by captive feral pigeons, provide a balanced diet, are reasonably uniform in size and composition, and have sufficient surface area so that an adequate coating of chemical can be deposited on the surface. Batches of sieved pellets (500 g) were mixed using an industrial food mixer and sprayed with the appropriate amount of compound dissolved in methanol + water (4 : 1 by volume, 175 ml). The concentration ranges to be tested were determined, where possible, from previous feeding studies (Crocker et al., 1993). This mixing process produced an evenly coated batch, with little or no damage to the pellets and no excess solvent. The pellets were air dried on a stainless steel tray and then sieved to remove any debris. Pilot trials had shown that the application of the methanol + water solvent alone did not affect the palatability of the pellets.

Sample Extraction. Eight samples (0.5 g) from each treatment preparation were ground to a fine powder and suspended in 20 ml of methanol + water solvent (4 : 1 by volume). The analyte was extracted by sonicating the sample for 30 min in a sealed centrifuge tube. After extraction, the sample was centrifuged (10,000 *g*, 20 min), and the supernatant stored at 4°C. This process was repeated to ensure complete extraction, the supernatants were pooled, and the total volume made up to 50 ml.

Sample Analysis. A Waters-Millipore HPLC system was used to separate and quantify the analyte. The system including a WISP autosampler and a Lambda-Max 480 UV detector. A prepacked analytical column (250 × 4.6 mm) and guard column (10 × 3.2 mm) of Kromasil RP-18 (5 μm) were employed for the separation. The mobile phase consisted of: (A) potassium dihydrogen orthophosphate buffer (pH 7.0, 20 mM), and (B) methanol. The elution profile was: 0–2 min 20% B; 2–24 min, 20–90% B; 24–28 min, 90% B; 28–29 min, 90–20% B; 29–32 min, 20% B, at a flow rate of 1.0 ml/min, column temperature of 35°C, and a col-

umn pressure of 14–20 MPa. Standards were weighed and dissolved in methanol + water (4 : 1 by volume) to give various concentrations within the range 0.0125–0.1 mg/ml. Calibration curves were plotted based on the linear regression analysis of peak area. All samples were filtered through a Millipore filter (FH, 0.45 μm). Sample filtrate (20 μl) was then injected onto the column and the concentration of the test compound determined from its calibration curve.

Pigeons. Feral pigeons were individually housed indoors at 16°C on a 12-hr light–dark cycle (08:30–20:30 hr). They were visually but not aurally isolated and were supplied with water, grit (Health Grit, Liverine, Grimsby, UK) and food (chicken layer pellets) ad libitum. They were weighed on entry to the room and three times a week for two to three weeks until the beginning of the experimental period. The experiment was not started until the birds maintained a stable body weight and any bird that lost more than 15% of its entry weight was excluded from the test. Throughout the experimental period the birds received water and grit ad libitum.

Pretrial Period. The birds were provided with chicken layer pellets (60 g) at 08:30 hr on three consecutive days. Their food consumption was measured at $1\frac{1}{2}$-hr intervals between 08:30 and 17:00 hr and after 24 hr (08:30 hr). The birds were weighed before and after the pretrial period. Between the pretrial and trial periods, the birds received chicken layer pellets ad libitum.

Trial (Short-Term No-Choice Test). The trial with treated food was carried out on three consecutive days in the week following the pretrial period. Five birds were randomly assigned to each treatment and received the same treatment on each day. The birds received 30 g of treated food at 08:30 hr and consumption was measured at $1\frac{1}{2}$-hr intervals between 08:30 and 17:00 hr. This procedure allowed patterns of food consumption to be monitored throughout the day and reduced the possibility of learned association between change of the food bowl or diet regimen. At 17:00 hr the treated food was replaced with 60 g of untreated food, which was removed at 08:30 hr the following morning. The 12-hr light–dark regime ensured that the birds had not fed or drunk for 12 hr before they received the treated food (feral pigeons will not feed in the dark; A. Dawson, 1993, personal communication). The birds were weighed before and after the three-day trial.

Throughout the trial and pretrial periods, equivalent measurements were taken from a bowl of chicken layer pellets (30 or 60 g, where appropriate) kept within the same room. This controlled for the effects of temperature and humidity on the weight of food.

Data Analyses: Food Consumption. The percentage change in consumption between the three-day pretrial and trial periods was calculated for each individual bird. A polynomial regression technique was then used to describe the dose–response relationship for each of the compounds under test. The best fitting model was determined by analyzing all 14 compounds together, fitting

polynomial terms, and allowing the parameters to vary for each compound. The relationship was best described by a second-order polynomial (improvement over linear fit, $F = 49.79$, df 1, 392, $P < 0.001$). Polynomial curves were used because they had the flexibility to fit all the compounds studied; more complex nonlinear curves, such as logistic curves, fitted some compounds better but did not give an acceptable fit with others.

From this regression line the R_{50} value (Starr et al., 1964; Watkins et al., 1995), analogous to the LD_{50} values used in toxicological studies and defined as the concentration of chemical required to reduce mean food consumption by 50%, was calculated. Confidence limits for the R_{50} values were determined using a bootstrapping technique (Efron and Tibshirani, 1993). In all cases, Tukey's Honestly Significant Difference tests were used to make pairwise comparisons and isolate significant differences ($P < 0.05$) between R_{50} values.

Molecular Modeling Procedure. Initial modeling of all structures and conformational analysis was carried out using CHEMX (Chemical Design Ltd., Chipping Norton, UK). Final conformational analysis was completed on the 14 compounds using Quanta96/CHARm (Molecular Simulations Inc., San Diego, California) to identify the correct lowest energy conformation of each compound. The bond angles, lengths, and torsions were also examined in the Cambridge Crystallographic Database (Cambridge Crystallographic Data Centre, Cambridge, UK). A grid search conformational analysis was carried out along with the four main torsion angles of the propenoic acid side chain as variables. In the minimum energy confrontation, the propenoic side chain was at a torsion angle of 19° to the aromatic ring (a compromise between conjugation and steric effects) and the polar hydrogen was found to extend out from the structure, synperiplanar with the carbonyl oxygen.

The bond angles, lengths, and torsions were then checked against values in crystal structures of cinnamic acid derivatives from the Cambridge Crystallographic Database. Fifty-five cinnamic acid derivatives with no substituents on the propenoic side chain were located, but the torsion angle between the aromatic ring and the propenoic acid side chain ranged between 1 and 8°. It is clear that the CHARm force field in our case has overestimated the steric effects by comparison with the conjugation in these bonds. For this reason, these torsion angles were adjusted to the mean experiment value of 3.6°, but all other dimensions were taken from the conformational analysis.

The molecular descriptors were generated from CHEMX, ClogP, Cerius2 QSAR descriptor and the TSAR software package (Oxford Molecular Ltd., Oxford, UK) with the Vamp QM calculation module (a MOPAC-type program using AM1 parameterization). The AM1 (Austin model 1) method (Dewar et al., 1985) is based on NDDO and MNDO, but the overall accuracy is considerably improved. The later PM3 method (Stewart, 1989) was not used, as it is thought that AM1 is more accurate for the generation of electronic charges (Dewar et al.,

Smith et al., 1992). In total, 110 independent descriptors were generated, about 30% of which were semiempirical molecular properties.

As many of the properties calculated had very different values or ranges, standardization was carried out to allow statistically meaningful analysis of the data, giving comparable weight to all the parameters involved. This is particularly important for partial least squares or principal component analysis. A correlation matrix with all descriptors was then constructed to identify any cross-correlated parameters. When two descriptors had a correlation of $r^2 > 0.70$, then the parameter that had a lower correlation with the log (R_{50}) values (dependent variable) was omitted. This process reduced the number of independent descriptors to 41.

Data Analysis: Structure–Activity Relationships. Various statistical analysis methods were employed to find a suitable descriptive equation, including multiple regression, partial least squares, and principal component analysis. A number of predictive equations were selected by these techniques, but all failed to give acceptable cross-validation indices [i.e., $r(CV)^2; > 0.8$]. The problem remained that there were too many possible combinations of independent variables to select manually an appropriate set for accurate prediction. Therefore, the genetic algorithm package in Cerius 3.0 was employed (Molecular Simulations Inc.). This method provides a fast and effective technique by which combinations of independent variables can be matched and predictive equations generated. The technique uses a genetic function algorithm (GFA), which provides the user with multiple models, not only linear polynomials but also higher order polynomials, splines, and Gaussians. However, as we were able to obtain high-quality equations with linear polynomials, it was not necessary to use the higher order equations. Use of linear equations also has the advantage that the equations are easier to interpret in a chemical sense. In the genetic algorithm technique an initial population of equations is generated with random bias. These are assessed on the basis of a fitness function, the best equations are selected and then mutated or combined with others via the genetic crossover procedure, and those with the best fitness function are retained and the process is repeated. Gradually over many mutations and crossovers, the best 100 equations are selected and reported together with their calculated correlation and cross-correlation coefficients. There are many choices of genetic algorithm defaults in the software and the most successful approach was to continually change the number of crossovers, the mutation probabilities, and/or the number of terms being added or removed from each equation. Generally around 5000–10,000 crossovers and a 50% mutation probability was used.

RESULTS

Dose–Response Relationships. The dose–response relationships were determined for 14 cinnamic acid derivatives (Figures 1 and 2). However, only 13 R_{50} values could be determined since the value for 3-methoxy-4-hydroxycinnamic

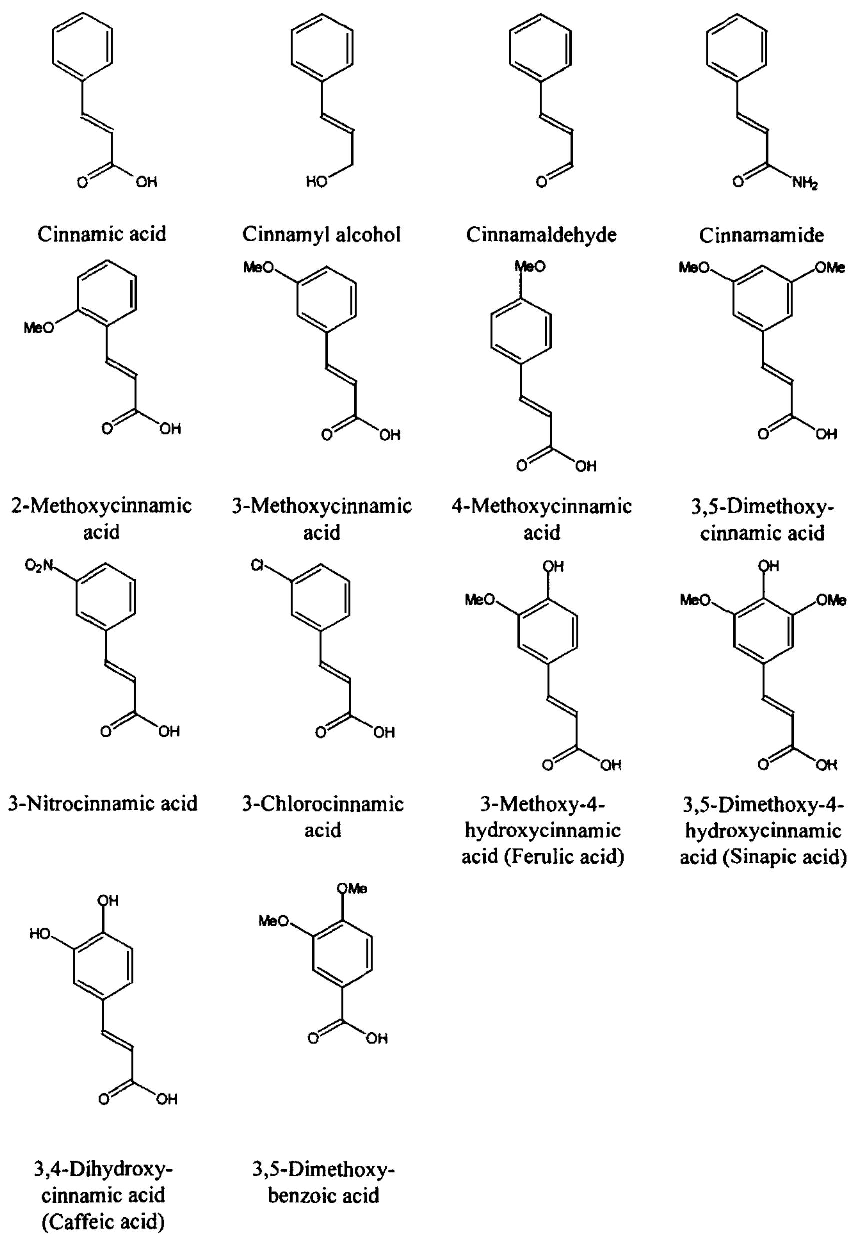


FIG. 1. Chemical structure of candidate cinnamic acid and derivative avian repellents.

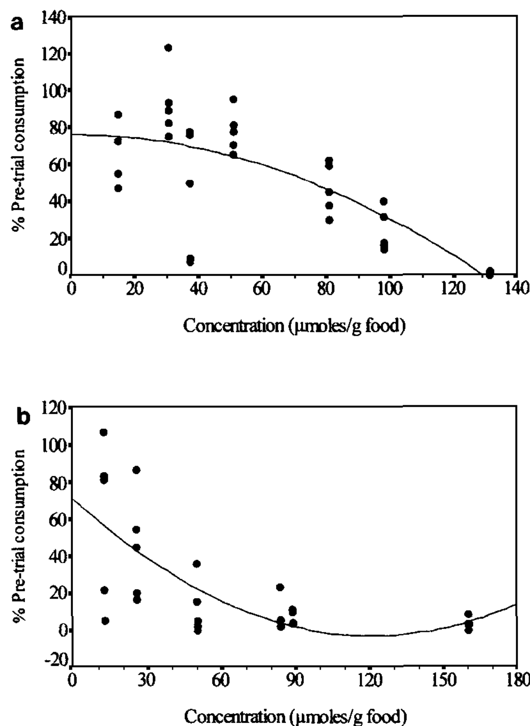


FIG. 2. Dose–response curves for the 14 cinnamic acid derivatives over the three-day, short-term, no-choice trial. The relationships between dose and response were described by second order polynomials. Solid dots depict mean consumption over three days expressed as a percentage of pretrial (normal) consumption; (a) cinnamic acid, (b) cinnamyl alcohol, (c) cinnamaldehyde, (d) cinnamamide, (e) 2-methoxycinnamic acid, (f) 3-methoxycinnamic acid, (g) 4-methoxycinnamic acid, (h) 3,5-dimethoxycinnamic acid, (i) 3-nitrocinnamic acid, (j) 3-chlorocinnamic acid, (k) 3-methoxy-4-hydroxycinnamic acid (ferulic acid), (l) 3,5-dimethoxy-4-hydroxycinnamic acid (sinapic acid), (m) 3,4-dihydroxycinnamic acid (caffeic acid), and (n) 3,5-dimethoxybenzoic acid.

acid (caffeic acid) could not be interpolated from the fitted curve without extrapolating beyond the observed doses (Table 1).

Post-hoc comparisons revealed that cinnamaldehyde was significantly more effective ($P < 0.05$), as measured by its R_{50} value, than all the other cinnamic acid derivatives with the exception of cinnamyl alcohol. Replacement of the carboxylic group in cinnamic acid with the aldehyde, amide and alcohol moieties of cinnamaldehyde, cinnamamide, and cinnamyl alcohol respectively, significantly increased repellency ($P < 0.05$; Table 2). The presence of electron-withdraw-

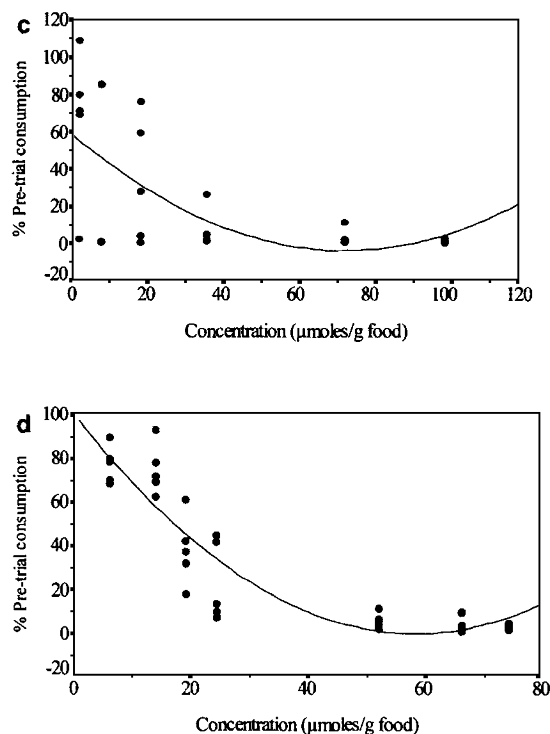


FIG. 2. Continued.

ing and electron-donating groups increases repellency with the meta-substituted (electron withdrawing) 3-methoxy-, 3-nitro-, and 3-chlorocinnamic acids being significantly more effective ($P < 0.05$) than the para-substituted (electron-donating) 4-methoxy-cinnamic acid.

Structure–Activity Relationships. The genetic function algorithm produced many linear regression models, but only one gave $r^2 > 0.90$ with relatively independent parameters, and this is reported below (Figure 3). This equation, which uses four electronic parameters [polarizability XY and YY, heat of formation (ΔH_f), and superdelocalizability (Sr)] had an r^2 of 0.980.

$$\log(R_{50}) = 2.1305 + 0.1200(\text{polarizability } XY) - 0.0296(\text{polarizability_}YY) - 0.0057(\Delta H_f) - 0.1498(Sr)$$

The predictive power of this equation was estimated using a cross-validation technique, the model being validated with up to three data points having been

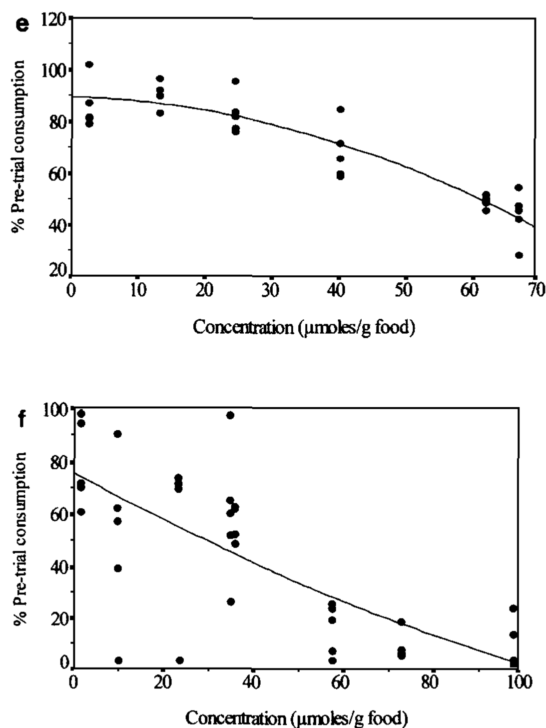


FIG. 2. Continued.

removed in each run, and the final value of $r(CV)^2$ was 0.938. Further validation of the predictive equation was undertaken by again using the genetic function algorithm to generate regression models on the original data, but after randomizing the R_{50} values relative to the independent descriptors. This was done in order to assess the tendency for the algorithm to detect spurious relationships in the data (Manly, 1991). Ten calculation runs were made with this randomized data. Poorly validated regression equations were generated; all resulting models produced poor cross-validation indices with $r(CV)^2 < 0.6$, in many cases < 0.2.

DISCUSSION

The results of any repellent study will be dependent, in part, upon the experimental paradigm selected. The standard technique used to evaluate repellency is to quantify food or water consumption in a choice or no-choice test. These two paradigms are likely to yield slightly different results: in the choice test the

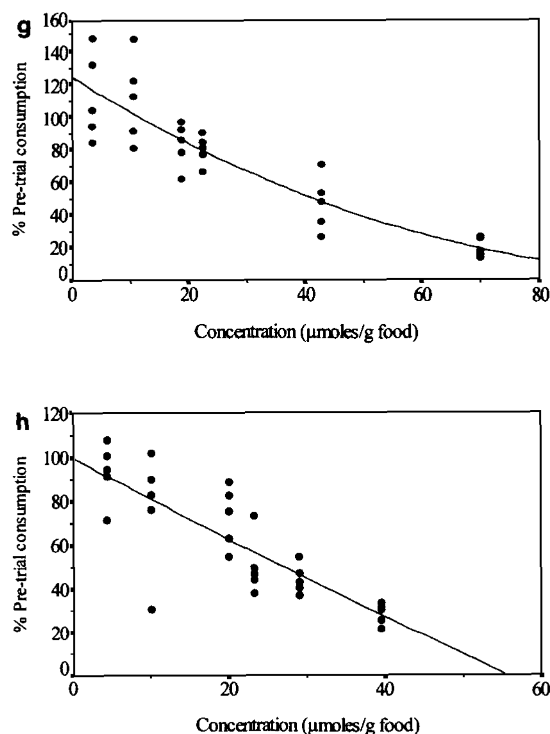
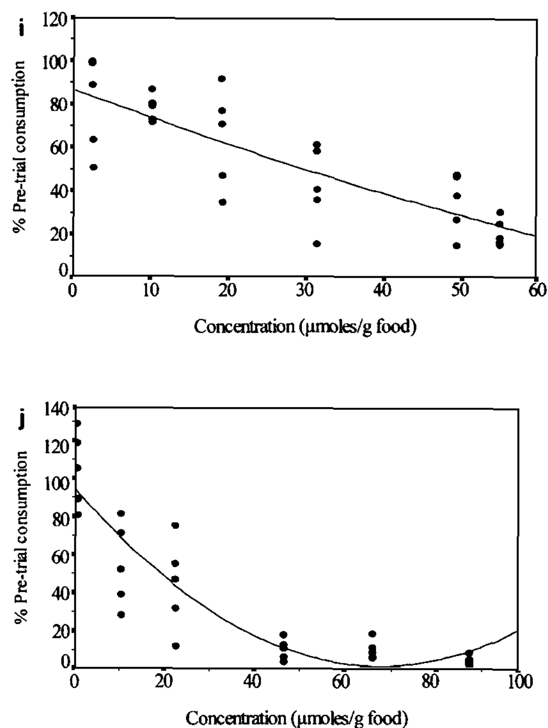


FIG. 2. Continued.

animal is presented with the choice between a palatable and unpalatable food, while in the no-choice test the animal can either consume the unpalatable food or go hungry. Thus the no-choice test is a more conservative measure of repellency and should therefore provide assurance as to its efficacy in field conditions where alternative foods are scarce. The current study was based on a short-term no-choice test carried out over three consecutive days. This test was designed to lessen the effects of food deprivation normally associated with the no-choice test, such as a reduction in food selectivity (Mason et al., 1989), but provide a more rigorous measure of repellency than that of the two-choice test.

The R_{50} value was interpolated from the dose–response relationships in order to categorize the potency of the cinnamic acid derivatives. The R_{50} values, analogous to the LD_{50} values used in toxicological studies, provide a useful and widely recognized single parameter estimate for comparison of repellent potencies in structure–activity studies (Schafer and Jacobson, 1983; Schafer et al., 1998). However, it should be noted that this value can only give a partial description of a compound's activity: an assessment of the slope, or sensitivity to



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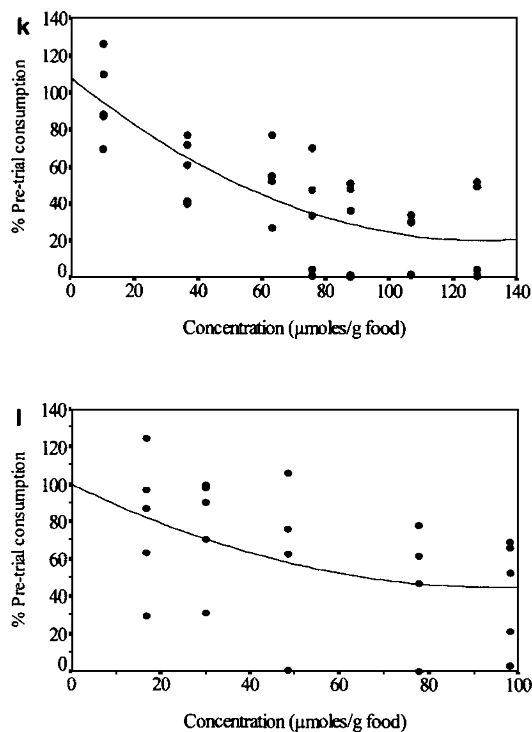
changes in concentration and the concentration at which maximum repellency is observed, can provide a fuller description of the compound's repellent properties (Clark and Shah, 1994).

From the inspection of the R_{50} values for the test compounds, two conclusions can be inferred. First and most significantly, the replacement of the —COOH group with other functionalities increases repellency: cinnamaldehyde > cinnamamide > cinnamyl alcohol > cinnamic acid. Second, that repellency is increased by the presence of electron-donating and electron-withdrawing groups, with the meta-substituted electron-withdrawing groups (e.g., 3-methoxy, 3-chloro, and the 3-nitroxy acids) playing the more significant role. Any quantitative regression model derived should therefore reflect these simple relationships.

Using the genetic algorithm, we have generated a quantitative model that can explain the structure–activity relationship for this series of cinnamic acid derivatives. The power of the model has been confirmed using both cross-validation and randomization tests. Further confirmation of the predictive power of this model will await analysis against an independent data set. In our main pre-



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diction equation the mechanisms behind the repellent activity are explained by a combination of four descriptors: heat of formation (ΔH_f), polarizability (XY and YY) and superdelocalizability (Sr). It is noteworthy that all these parameters are electronic, indicating that changes in electronic distribution within cinnamic acid structures are crucial for activity.

The first parameter, the heat of formation, describes the sum of four energy states: the energy required to ionize the valence electrons of the atoms involved, $E_{el}(A)$; the heat of atomization, $\Delta H_f(A)$; electronic energy; and nuclear energy. This descriptor reflects the increasing number of electron-withdrawing groups being added to the cinnamic acid parent structure: as more electron withdrawing groups are added to the ring, there are more atoms contributing to the energy terms and consequently the heat of formation increases. Although this relationship remains valid for our small set of compounds, it cannot necessarily be applied to more varied molecules.

The second and third parameters, the polarizability XY and YY are component vectors to the overall polarizability of the molecule. The tensor term XY

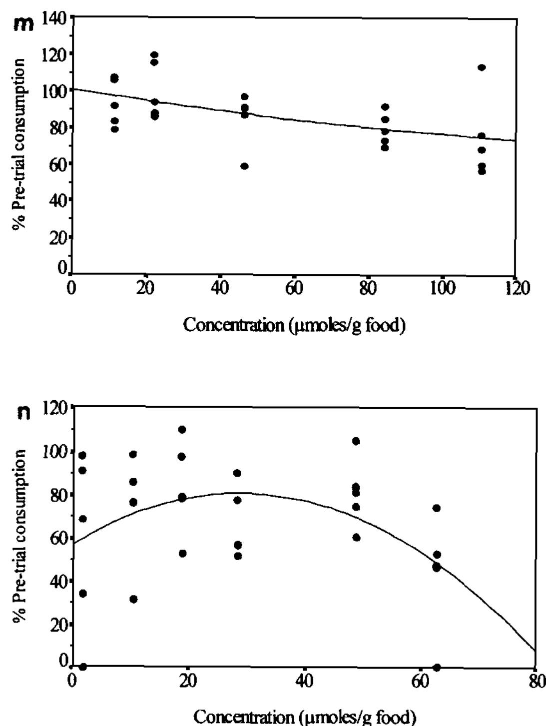

FIG. 2. Continued.

describes the ability of the molecule to be polarized in the y direction as a charge is exerted across the z axis (Figure 4). This reflects a change in charge distribution across the acid group, best illustrated by the marked increase in activity observed when the —COOH functional group of cinnamic acid is replaced by the amide of cinnamamide.

The last descriptor, superdelocalizability was originally derived as an index of reactivity of aromatic hydrocarbons. The index is based on the idea that early interaction of the molecular orbitals of reactants can be regarded as a mutual perturbation, the relative energies of orbitals changing together and maintaining a similar degree of overlap as reactants approach each other. The index is calculated by the sum:

$$Sr = 2\sum_{j=1}^{m} \frac{c_{jr}^2}{e_j}$$

TABLE 1. PARAMETER VALUES AND CURVE-FIT DETAILS FOR DOSE-RESPONSE RELATIONSHIPS OF CANDIDATE BIRD REPELLENTS[a]

Compound	R_{50}	SE	95% confidence limits	
			Lower	Upper
Cinnamic acid	75.76	5.6	64.1	85.1
Cinnamyl alcohol	19.35	13.3	0	33.6
Cinnamaldehyde	5.12	8.9	0	14
Cinnamamide	17.14	1.1	15.1	19.5
2-Methoxycinnamic acid	61.52	1.4	58.6	64
3-Methoxycinnamic acid	29.81	5.1	18.1	38.6
4-Methoxycinnamic acid	41.68	3.5	35.3	48.7
3,5-Dimethoxycinnamic acid	27.21	1.6	23.4	29.8
3-Nitrocinnamic acid	29.61	4.5	21.5	38.3
3-Chlorocinnamic acid	19.82	2	15.4	23.7
3-Methoxy-4-hydroxycinnamic acid	53.27	6.1	42.1	67.1
3,5-Dimethoxy-4-hydroxycinnamic acid	66.95	22.4	32.2	108.4
3,4-Dihydroxycinnamic acid	[b]			
3,5-Dimethoxybenzoic acid	61.94	7.8	54.5	89.6

[a]Confidence limits for the R_{50} values were determined using a bootstrapping technique.
[b]3-Methoxy-4-hydroxycinnamic acid (caffeic acid) could not be interpolated from the fitted curve without extrapolating beyond the observed doses.

Here the term Sr is the superdelocalizability at position r, e_j is the bonding energy coefficient in the jth molecular orbital (eigenvalue), c is the molecular orbital coefficient at position r in the HOMO (highest occupied molecular orbital) and m is the index of the HOMO. This descriptor has been found to correlate highly to the activity of different sets of compounds in many studies (Purdy, 1991; Nandihalli et al., 1992; Karabunarlie et al., 1996).

The equation establishes the electronic effects of substitution on the cinnamic acid aromatic ring as important factors for the resulting activity, corroborating earlier observations. Polarizability and superdelocalizability descriptors highlight the shift in charge distribution across the molecule as the substituents are changed. Due to resonance or inductive effects through the conjugated cinnamic acid (3-phenyl-propenoic acid) skeleton, changes in the position (ortho, meta, or para) or substituent type will have a marked influence on the distribution of electrons throughout this electron-rich system and thus the repellency of the compound.

Having developed a quantitative model for this series of molecule, the next logical step is to design compounds that combine and exaggerate those characteristics that underlie repellency. The marked increase in repellency is observed when the acid functional group is replaced by a less polar one or when electron

TABLE 2. PAIRWISE COMPARISONS BETWEEN R_{50} VALUES BASED ON STANDARD ERRORS FROM BOOTSTRAPPING ANALYSIS[a]

	Cinnamic acid	Cinnamyl alcohol	Cinnam-aldehyde	3-Methoxy-4-hydroxy CA	3,5-Dimethoxy-4-hydroxy CA	Cinna-mamide	3,5-Dimethoxy CA	4-Methoxy CA	3,5-Dimethoxy BA	3-Nitro CA	2-Methoxy CA	3-Chloro CA	3-Methoxy CA
Cinnamic acid													
Cinnamyl alcohol	b												
Cinnamyl aldehyde	b	NS											
3-Methoxy-4-hydroxy CA	b	b	b										
3,5-Dimethoxy -4-hydroxy	NS	b	b	NS									
Cinnamamide	b	NS	b	b	b								
3,5-Dimethoxy CA	b	NS	b	b	b	b							
4-Methoxy CA	b	b	b	b	b	b	b						
3,5-Dimethoxy BA	b	b		NS	NS	b	b	b					
3-Nitro CA	b	NS	b	b	b	b	NS	b	b				
2-Methoxy CA	b	b	b	NS	NS	b	b	b	NS	b			
3-Chloro CA	b	NS	b	b	b	NS	b	b	b	b	b		
3-Methoxy CA	b	NS	b	b	b	b	NS	b	b	NS	b	b	

[a]Significant differences were isolated using Tukey's Honestly Significant Difference tests.
[b]Significance at the 5% level; NS, nonsignificant differences. CA = cinnamic acid, BA = benzoic acid.

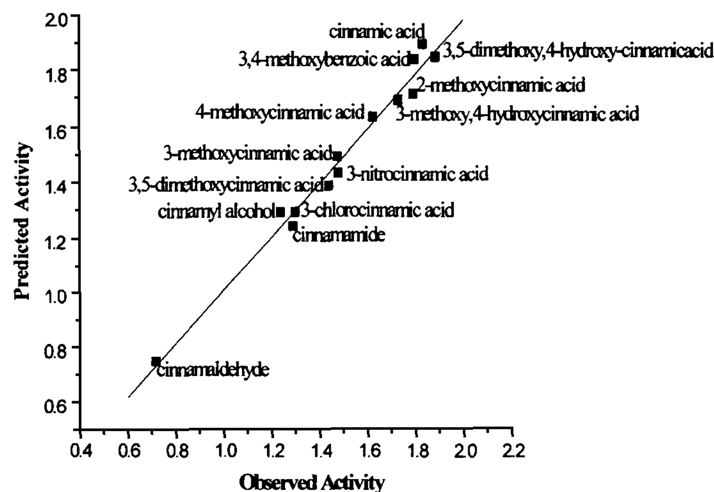

FIG. 3. Quantitative structure–activity model for candidate bird repellents. Plot of observed versus predicted log R_{50} values.

withdrawing groups are substituted on the aromatic ring. These predictions were confirmed by the model: 3-chlorocinnamaldehyde should be a potentially strong repellent. The model corroborates this prediction with a log R_{50} value of 0.59, although final validation will only be obtained when this compound and others are made and tested successfully.

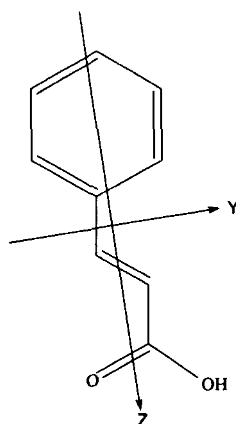

FIG. 4. The z and y axes of the electronic parameter tensors.

CONCLUSION

A simple regression equation has been derived and statistically validated that confirms activities of our test compounds and can predict the activity of putative cinnamic repellents. This is the first published quantitative structure–activity model for avian repellents and as a result we can now begin to predict which cinnamic acid derivatives (e.g., 3-chlorocinnamaldehyde) should make effective repellents. Quantitative structure–activity models have been developed by Clark and colleagues (unpublished) to explain the repellency of acetophenone and anthranilate derivatives using charge and topological descriptors. Further experimentation is required to assess the commonality of these models and to test their power in selecting new repellents from a more diverse set of compounds and for a range of avian species. However, this modeling approach promises to provide a more efficient and economic method for prospecting chemical databases for new effective bird repellents.

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GREEN LEAF VOLATILES INTERRUPT PHEROMONE RESPONSE OF SPRUCE BARK BEETLE, *Ips typographus*

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Abstract—A synthetic mixture of nine green leaf volatiles (GLVs) including linalool was tested on antennae of *Ips typographus* (L.) with coupled gas chromatographic–electroantennographic detection (GC-EAD). Strong responses were found to 1-hexanol, (*Z*)-3-hexen-1-ol, and (*E*)-2-hexen-1-ol. Weak responses were recorded to (*E*)-3-hexen-1-ol, (*Z*)-2-hexen-1-ol and linalool, while hexanal, (*E*)-2-hexenal and (*E*)-3-hexenyl acetate elicited no EAD responses. In a laboratory walking bioassay, the attraction of *I. typographus* females to a synthetic pheromone source was significantly reduced when a mixture of the three most EAD-active GLV alcohols was added to the source. Further reduction in response was obtained when these three alcohols were combined with verbenone (Vn). In field trapping experiments, a blend of 1-hexanol, (*Z*)-3-hexen-1-ol, and (*E*)-2-hexen-1-ol reduced *I. typographus* trap catches by 85%, while ca. 70% reduction of trap catch was achieved by Vn or a blend of (*E*)-3-hexen-1-ol, (*Z*)-2-hexen-1-ol, and linalool. The strongest disruptive effect was found when Vn plus a blend of the three most EAD active GLV alcohols was added to the pheromone trap (95% catch reduction). Adding the blend of the three most EAD active alcohols to pheromone-baited traps significantly reduced the proportion of males captured. These three GLV alcohols were also disruptive in the laboratory and in the field when tested individually. Hexanal, (*E*)-2-hexenal, and (*Z*)-3-hexenyl acetate were inactive both in the lab and in the field. Our results suggest that these nonhost green leaf alcohols may explain part of the host selection behavior of conifer-attacking bark beetles and may offer a source of inhibitory signals for alternative management strategy for forest protection.

Key Words—*Ips typographus*, Coleoptera, Scolytidae, semiochemicals, pheromones, green leaf volatiles, GLV, (*Z*)-3-hexen-1-ol, 1-hexanol, (*E*)-2-hexen-1-ol, (*E*)-3-hexen-1-ol, (*Z*)-2-hexen-1-ol, hexanal, (*E*)-2-hexenal, (*Z*)-3-hexenyl acetate, linalool, verbenone.

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INTRODUCTION

Suitable hosts of bark beetles are widely scattered throughout mixed species forests and are distributed unevenly in space and time (Atkins, 1966). Therefore, bark beetles commonly utilize specialized and complex semiochemical messages to locate suitable breeding material (Borden, 1985; Schlyter and Birgersson, 1999). In seeking suitable hosts, bark beetles encounter and reject many unsuitable hosts and nonhost trees (Borden, 1996). Rejection could be based on a lack of certain host volatile characteristics and/or a negative response to some nonhost stimuli. Ambrosia beetles attacking on broad-leaved trees, *Trypodendron domesticum* (Oliver) and *Xyleborus (Anisandrus) dispar* (F.), respond negatively to α-pinene, one of the major monoterpene constituents of both Scots pine, (*Pinus sylvetris* L.), and Norway spruce [*P. abies* (L.)] (Nijholt and Schönherr, 1976; Schroeder and Lindelöw, 1989). In the presence of certain nonhost substances, *Scolytus multistriatus* Marsham rejected host-tree twigs suitable for maturation feeding (Gilbert and Norris, 1968).

In central Sweden, attraction of both *Tomicus piniperda* (L.) and *Hylurgops palliatus* (Gyll.) to ethanol-baited traps was reduced by the presence of nonhost bolts from birch, *Betula pendula* Roth, or aspen, *Populus tremula* L. (Schroeder, 1992). Studies with the North American species, *Dendroctonus frontalis* Zimmermann, *Ips grandicollis* (Eichhoff) and *I. avulsus* (Eichhoff), showed that bark beetles might avoid nonhost trees because their response to pheromone was inhibited by several compounds among the green-leaf volatiles (GLVs) (Dickens et al., 1991, 1992). GLVs are mainly six-carbon primary alcohols, aldehydes, and derivative esters that are commonly found in green plants (Visser, 1986; Whitman and Eller, 1990; Dickens et al., 1991, 1992). In Europe, Schlyter et al. (1995) found that *Ips typographus* (L.), *I. duplicatus* (Sahlb.), and *T. piniperda* were inhibited by a blend of six GLVs including linalool. Recently, it was shown that attraction of conifer bark beetles, *D. ponderosae* Hopkins, *D. rufipennis* Kirby, and *D. brevicomis* LeConte in Canada, and *T. piniperda* in USA, to their pheromone or kairomone components were reduced by the GLVs (Wilson et al., 1996; Borden et al., 1997; Poland et al., 1998; Poland and Haack, 2000). The green leaf alcohols also have been shown in trapping experiments to disrupt the response to aggregation pheromones by conifer-infesting ambrosia beetles (Scolytidae) in British Columbia, including *Tryp. lineatum* (Oliv.) (Borden et al., 1997), *Gnathotrichus sulcatus* (LeConte), and *G. retusus* (Deglow and Borden, 1998a,b). Some nonhost bark volatiles, such as *trans*-conophthorin, have been found to be antenally active and behaviorally disruptive in *D. ponderosae* and *D. pseudotsugae* Hopkins by Huber et al. (1999), and in *I. typographus* by Zhang et al. (2000, unpublished data). Guerrero et al. (1997) reported that benzyl alcohol identified in the eucalyptus callus was antennally active and might reduce attraction of *Tomicus destruens* (Woll.) to host logs. Single-cell responses

to unknown compounds from birch bark have been demonstrated in *Tryp. lineatum* and *I. typographus* (Tømmerås, 1989, Tømmerås and Mustaparta, 1989).

European birches, *B. pendula* and *B. pubescens* Ehrh., and aspen, *P. tremula*, are often found in mixed stands with Norway spruce in Scandinavia. Recent studies showed that attraction of *I. typographus* and *Pityogenes chalcographus* (L.) to pheromone traps was significantly reduced by the presence of fresh birch bark and leaves (Byers et al., 1998). Volatiles from intact branches (with leaves) of nonhost trees, *B. pendula*, *B. pubescens*, *P. tremula*, and *Sambucus nigra* L., have been collected by headspace sampling and analyzed by GC-MS (Zhang et al., 1999). In GC-EAD analyses of *B. pendula* and *B. pubescens* leaf volatiles, *I. typographus* antennae strongly responded to 1-hexanol, (*Z*)-3-hexen-1-ol, and (*E*)-2-hexen-1-ol, the major green leaf alcohols released from intact birch leaves (Zhang et al., 1999).

Our objectives were to test the electrophysiological and behavioral activities of GLVs in the spruce bark beetle, *I. typographus.*

METHODS AND MATERIALS

Electrophysiological Analysis: GC-EAD

A 3-μl sample of synthetic GLV mixture including hexanal, (*E*)-2-hexenal, (Z)-3-hexenyl acetate, 1-hexanol, (Z)-3-hexen-1-ol, (*E*)-3-hexen-1-ol, (Z)-2-hexen-1-ol, (*E*)-2-hexen-1-ol, and linalool with 10 ng each in 1 μl of hexane was injected splitless into an HP 6890 GC equipped with an HP-INNOWAX column (30 m $\times$ 0.25 mm $\times$ 0.25 μm) and a 1 : 1 effluent splitter that allowed simultaneous flame ionization detection (FID) and EAD of the separated volatile compounds. Hydrogen was used as the carrier gas, and the injector temperature was 220°C. The column temperature was 30°C for the first 3 min, rising to 200°C at 10°C/min, and held for 2 min. The outlet split for the EAD was inserted into a humidified airstream (0.5 m/s) flowing over an *I. typographus* antennal preparation. A glass capillary indifferent electrode filled with Beadle-Ephrussi Ringer, and grounded via a silver wire, was inserted into the severed beetle's head attached to the antennae. A similar recording electrode connected to a high-impedance DC amplifier with automatic baseline drift compensation was placed in contact with the distal end of the antennal club. The signal was stored and analyzed on a PC equipped with an IDAC card and the program EAD ver. 2.3 from Syntech, Hilversum, The Netherlands.

Walking Bioassay

Behavioral responses were tested in the laboratory with an open area walking bioassay olfactometer (Byers and Wood, 1981; Schlyter et al., 1995) at

24–25°C and 200 lux. *I. typographus* female adults (preselected) were released downwind in a laminar flow of air at ca. 1 m/sec speed in the center of the odor plume generated by one or two capillaries with test materials. The flow of pure air was obtained by charcoal filtering reduced-pressure compressed air let into a baffle with spaced 2-mm holes. The form and speed of airflow were observed by puffs of cigarette smoke. Beetles were released in groups of 10–20 in the center of the area opposite to the odor source. Those beetles walking to within a 2-cm radius of the source in 3 min were scored as responding. Beetles leaving the larger circle in other directions were scored as not responding, but were given a second try. These beetles were tested against a mix of synthetic pheromone components, 2-methyl-3-buten-2-ol (MB) and 4*S*-*cis*-verbenol (cV) at 150 : 1 as a neat solution evaporating from a 50-μl Microcaps with one end sealed by dental wax (0.80 mm ID), as a positive control. Verbenone (Vn) was added in a low dose to the pheromone neat solution at a MB/cV/Vn ratio of 150 : 1:0.1 as a negative control (Bakke, 1981; Schlyter et al., 1995). GLVs in blends of three to nine compounds (grouped by their GC-EAD activity; see Figure 2 and Table 2, below, for details) were added in a separate capillary placed downwind in contact with the pheromone dispenser. Release rates were estimated as follows by following the retreat of the meniscus over time: for MB and cV 1.7×10^{-6}, for Vn 0.2×10^{-8}, and for GLV mix 2.3×10^{-7} (g/min) (Schlyter et al., 1995). For the GLV mix, the proportions in the blend were adjusted based on release rates of individual components to give an equal rate of release of each component.

Adults of *I. typographus* for analysis and bioassay were obtained from continuous cultures maintained on Norway spruce bolts (Schlyter and Anderbrant, 1993). For GC-EAD analysis, beetles were taken from generation 94 (T94) of a strain originally collected from Lardal, southern Norway in 1983, and from a second generation (W2) of a strain collected in Torsby, central Sweden, 1997. The antennal responses of both sexes (Schlyter and Cederhom, 1981) were measured. In the walking bioassay test, female beetles were taken from generations T95 and W3 of both strains. A 24-hr period of flight exercise under the conditions of 25°C and 4000 lux was given to the beetles before the walking test.

Field Trapping

Group GLVs Test. This test was conducted in an old spruce clear-cut, near Asa Forest Research Station, SLU, Småland, Sweden (57°08′N, 14°45′E) during May 15–June 25, 1998. Nine black drainpipe traps (type N79) were set up in a line with 10 m between traps. The blends of GLVs were grouped and tested based on their GC-EAD activity. Traps together with dispensers were rotated after each replicate (when ≥30 beetles were caught in the best trap) in a randomized Latin-square design (Byers, 1991).

Individual GLVs Test. The inhibition effects of individual GLVs were tested

in a spruce-pine mixed stand, ca. 5 km south of the group GLVs test site, during June 8–July 11, 1998. Twelve 12-unit multiple-funnel traps (Phero-Tech, Delta, British Columbia, Canada) hung from 1.5 m poles, were set up in line with 10 m between traps and ≥5 m from spruce trees. Dispensers were put under one inverted 250-ml plastic cup painted with light grey, with bait positions rotated after each replicate (When ≥25 beetles were caught in the best trap) in a randomized Latin-square design.

All chemicals, sources, release devices, and release rates are listed in Table 1. The release rate of verbenone was lower than that for maximal inhibition (Schlyter et al., 1989) to allow the effects of GLVs and their synergism with Vn to be detected. In all the field experiments, three control treatments were used to assess the effectiveness of candidate GLVs, as follows: (1) the positive control, i.e., synthetic pheromone bait (MB + cV); (2) the negative control, consisting of the synthetic pheromone lure plus a dispenser with the known inhibitor Vn (Schlyter et al., 1989); and (3) the blank control, unbaited trap.

Statistical Analysis

Statistical analysis was done by one-way ANOVA of untransformed (GC-EAD) and arcsin $\sqrt{p}$ transformed (walking bioassay and field trapping tests) data, followed by Duncan's multiple range test (SPSS 8.0 for Windows). In all cases $\alpha = 0.05$. Because of considerable variations in numbers of beetles caught (in the field) between replicates and experiments, the counts were converted to the proportion (p) of total captured beetles within each replicate. The sex ratios for the treatments within each experiment were compared with 95% binomial confidence intervals (Byers and Wood, 1980).

RESULTS

GC-EAD Responses. GC-EAD analysis of a synthetic GLV mixture containing two 6-carbon aldehydes, one acetate, (*Z*)-3-hexenyl acetate, and five 6-carbon alcohols plus linalool with the same amount (30 ng each injected, thus ca. 15 ng each in the outlet directed to the antenna after separation from GC), showed that both male and female *I. typographus* antennae responded strongly to (*Z*)-3-hexen-1-ol, 1-hexanol, and (*E*)-2-hexen-1-ol, which commonly exist in nonhost leaves (Zhang et al., 1999) (Figure 1). Weaker responses were recorded to (*E*)-3-hexen-1-ol, (*Z*)-2-hexen-1-ol, and linalool (Figure 1). No responses were found to the aldehydes or the acetate. Differences in EAD responses between these three groups were significant $P < 0.05$; $N = 8$), but not within the groups.

Walking Bioassay Test in Laboratory. After 24 hr of flight exercise, ca. 60% of *I. typographus* females responded by walking to the dispenser containing

TABLE 1. CHEMICALS, RELEASE RATES, AND DISPENSERS USED IN FIELD-TRAPPING EXPERIMENTS

Attractant/inhibitor	Source	Purity (%)	Release rate (mg/day)[a] ±95% CL	Dispenser
Pheromone				
2-Methyl-3-buten-2-ol (MB)	Aldrich	97	57 ± 0.8	2 ml in #733 PE vial[b] with 2-mm-diam. hole in lid
(4*S*)-(−)-verbenol (cV)	Borregaard	98	1.0 ± 0.05	40 mg in hard PE vial[c] with 9-mm-diam. hole in lid
Inhibitors				
(GLVs and Vn)				
Hexanal	Aldrich	98	13.5 ± 4.5	200 μl in #730 PE vial[d] with 2-mm-diam. hole in lid
(*E*)-2-hexenal	Aldrich	99	6.9 ± 2.0	200 μl in #730 PE vial with 2-mm-diam. hole in lid
(*Z*)-3-hexenyl acetate	Lancaster	99	9.4 ± 0.17	200 μl in an open #730 PE vial
1-Hexanol	Aldrich	98	4.1 ± 0.13	200 μl in an open #730 PE vial
(*Z*)-3-Hexen-1-ol	Aldrich	98	6.3 ± 0.11	200 μl in an open #730 PE vial
(*E*)-2-Hexen-1-ol	Aldrich	97	5.1 ± 0.09	200 μl in an open #730 PE vial
(*E*)-3-Hexen-1-ol	Aldrich	98	5.9 ± 0.09	200 μl in an open #730 PE vial
(*Z*)-2-Hexen-1-ol	Acros	95	5.1 ± 0.08	200 μl in an open #730 PE vial
Linalool	Aldrich	97	1.3 ± 0.04	200 μl in an open #730 PE vial
Verbenone (Vn)	Aldrich	99	0.5 ± 0.02	150 μl in an open #730 PE vial

[a]Measured in a mini-wind tunnel at 20–21°C and 0.7 m/sec for 10 days. Rate calculated as the slope ± its 95% confidence limits of the regression of weight loss versus time.
[b]Polyethylene vial with 20-mm-diam., 29 mm inner height.
[c]3 ml-hard Polyethylene vial with 13-mm-diam., 24 mm inner height.
[d]Polyethylene vial with 6-mm-diam., 29 mm inner height.

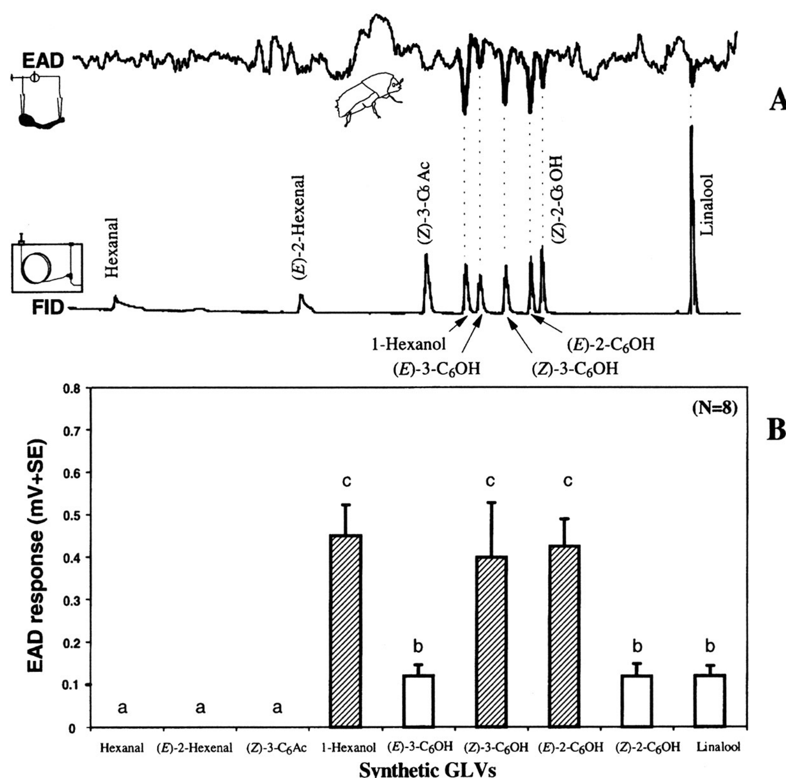

FIG. 1. GC-EAD response of *Ips typographus* to a mixture of nine synthetic GLVs including linalool. (A) Representative GC-EAD trace; each GC-peak represents ca. 15 ng. (B) Summary of 8 GC-EAD runs. Bars with the same letter are not significantly different ($P > 0.05$) by ANOVA followed by Duncan's multiple-range test.

pheromone alone, and there was almost no attraction to the blank control (with no pheromone or other odors). Relative responses to the test stimuli were calculated by dividing by the percent response to pheromone alone (Figure 2A). A significant reduction of attraction was found by the mixture of the three most EAD-active alcohols [3OH-A: 1-hexanol, (*Z*)-3-hexen-1-ol, and (*E*)-2-hexen-ol], and the mixture of all nine GC-EAD tested compounds (9-EAD) when added to the pheromone source. It was similar to the inhibition caused by Vn, the negative control (Figure 2A). Further reductions in female attraction to the pheromone dispenser were obtained when the mixture of all six alcohols (6-OH) or combination of Vn and 3OH-A were present (Figure 2A). However, the mixture of hexanal, (*E*)-2-hexenal, and (*Z*)-3-hexenyl acetate (2Ald-Ac in Figure 2 and Table

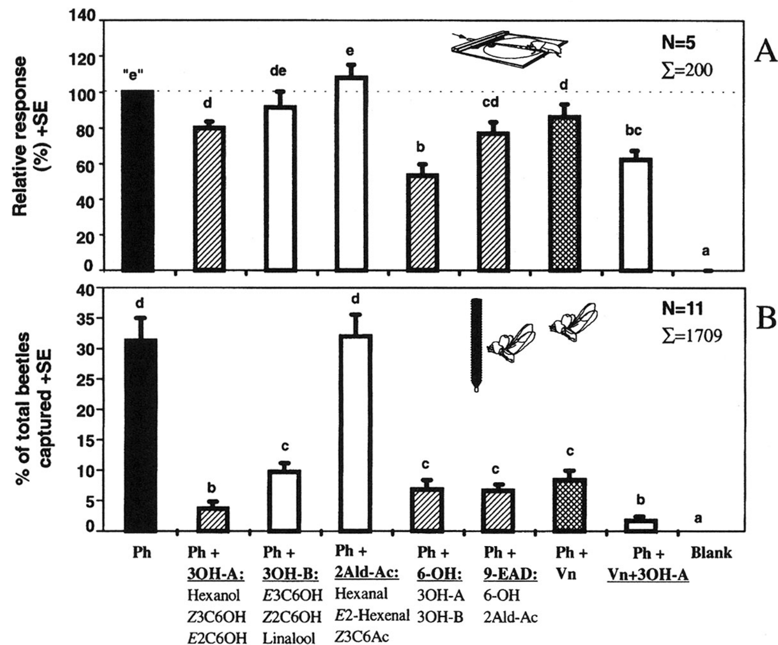

FIG. 2. Response of *Ips typographus* to GLVs and verbenone added to its pheromone source. (A) Female walking bioassay, March 1998; the relative responses (%) = (percent responses to test stimuli/percent response to pheromone alone) × 100; hypothesis testing (ANOVA and range-test) was done on the absolute response frequencies. (B) Field trapping experiment, Asa, Sweden, May–June 1998, with 11 replicates and a total of 1709 beetles captured. Bars with the same letter are not significantly different ($P > 0.05$) by ANOVA on arcsine $\sqrt{p}$ followed by Duncan's multiple-range test.

2) did not decrease attraction of *I. typographus* to the pheromone dispenser (Figure 2A). The same is also true for the mixture of three alcohols showing weaker EAD activity, i.e., 3OH-B: (*E*)-3-hexen-1-ol, (*Z*)-2-hexen-1-ol, and linalool.

Reductions in female orientation to pheromone source also were caused by the single green leaf alcohols, 1-hexanol, (*Z*)-3-hexen-1-ol, and (*E*)-2-hexen-1-ol, but not by the EAD inactive aldehyde, hexanal (Figure 3).

Field Trapping. In the group GLV test, a total of 1709 *I. typographus* were captured during 11 replicates. The blend of the two aldehydes plus the acetate (2Ald-Ac) did not reduce trap catches. In contrast, the blend of 3OH-A resulted in an 85% reduction in the number of *I. typographus* captured compared to that in traps baited with pheromone alone (Figure 2B). Significant trap catch reductions ranging from 68% to 78% were found when the GLV groups, 3OH-B, 6-OH, 9-EAD, or Vn were added to the pheromone traps. No significant dif-

TABLE 2. PROPORTION OF *Ips typographus* MALES CAPTURED

Experiment and Treatment[a]	% Males	95% CI	Total catch
Group GLV with pipe traps ($\sum = 1709$, $N = 11$), May–June 1998			
Ph	29.6	26.2–33.4	614
Ph + 3OH-A	15.2	8.4–25.7[b]	66
Ph + 3OH-B	20.8	15.4–27.6	168
Ph + 2Ald-Ac	31.6	27.6–35.8	491
Ph + 6-OH	21.2	13.8–31.0	85
Ph + 9EAD	22.5	15.5–31.6	102
Ph + Vn	21.2	15.4–28.4	151
Ph + Vn + 3OH-A	18.8	8.9–35.3	32
Blank	—		0
Individual GLV with multiple funnel traps ($\sum = 2128$, $N = 12$), June–July 1998			
Ph	37.0	30.9–43.6	219
Ph + 1-hexanol	19.6	13.3–28.0[b]	112
Ph + (*Z*)-3-hexen-1-ol	27.3	20.4–35.4	132
Ph + (*E*)-2-hexen-1-ol	22.9	16.1–31.7	109
Ph + (*E*)-3-hexen-1-ol	28.6	22.0–36.2	154
Ph + (*Z*)-2-hexen-1-ol	32.9	26.1–40.6	158
Ph + linalool	35.5	30.4–41.1	301
Ph + hexanol	32.9	28.0–38.3	316
Ph + (*E*)-2-hexenal	36.5	30.7–42.7	244
Ph + (*Z*)-3-hexenyl acetate	35.5	30.0–41.5	262
Ph + Vn	24.6	17.6–33.2	114
Blank	42.9	15.8–75.0	7

[a]Ph: aggregation pheromone bait [(4*S*)-(−)-*cis*-verbenol and 2-methyl-3-buten-2-ol]; Vn: verbenone; 3OH-A (EAD strongly active alcohols): 1-hexanol, (*Z*)-3-hexen-1-ol, and (*E*)-2-hexen-1-ol; 3OH-B (EAD weakly active alcohols): (*E*)-3-hexen-1-ol, (*Z*)-2-hexen-1-ol, and linalool; 2Ald-Ac: hexanal, (*E*)-2-hexenal, and (*Z*)-3-hexenyl acetate; 6-OH: 3OH-A + 3OH-B; 9EAD: 6-OH + 2Ald-Ac.
[b]Significantly different from the pheromone alone, at $P < 95\%$ (binomial confidence intervals).

ferences between these four treatments were found. The strongest disruptive effect resulted from the addition of the combination of Vn with 3OH-A to the pheromone trap, which caused 95% reduction in trap catch (Figure 2B). The proportion of males was reduced by 50% from 1 : 2.37 (M/F) in the pheromone control trap to 1 : 5.6 in the 3OH-A plus pheromone trap (Table 2). The combination of Vn and 3OH-A did not significantly reduce the proportion of males (Table 2).

In the single GLV test, a total of 2128 *I. typographus* were captured during 12 replicates. Neither of the individual GLV alcohols in the 3OH-B group, nor

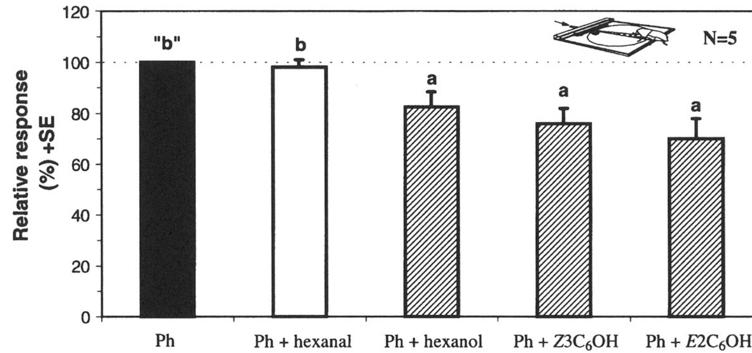

FIG. 3. Relative response of walking *Ips typographus* females to individual GLVs added to its pheromone, April 1998. Bars with the same letter are not significantly different ($P > 0.05$) by ANOVA on arcsin $\sqrt{p}$ followed by Duncan's multiple-range test.

the members of 2Ald-Ac alone significantly reduced trap catches (Figure 4). In contrast, the trap catches were significantly disrupted by Vn and all the three individual alcohols belonging to group 3OH-A, i.e., 1-hexanol, (*Z*)-3-hexen-1-ol, and (*E*)-2-hexen-1-ol. The catch reduction ranged from 41% to 50% (Figure 4). Moreover, the percentage of males captured in the trap with 1-hexanol was

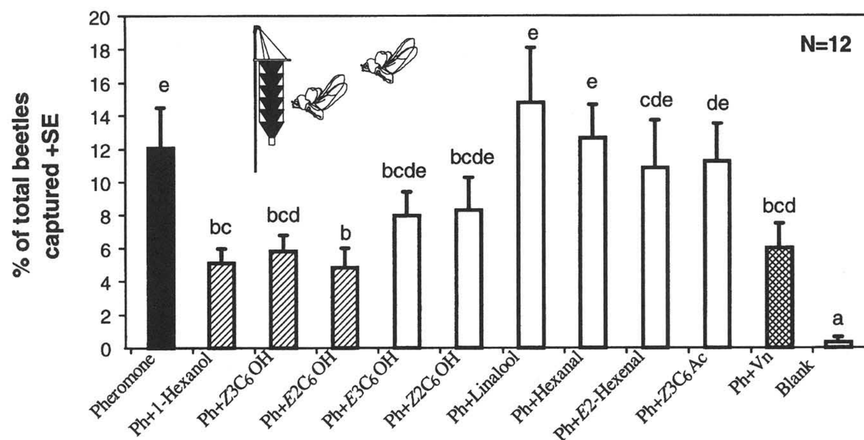

FIG. 4. Effects of individual GLVs on the capture of *Ips typographus* in pheromone-baited multiple funnel traps, Asa, Sweden, June-July, 1998, 12 replicates, total catch = 2128. Bars with the same letter are not significantly different ($P > 0.05$) by ANOVA on arcsin $\sqrt{p}$ followed by Duncan's multiple-range test.

significantly lower than that from the pheromone control trap, which indicated a stronger disruptive effect on males than on females (Table 2). No statistically significant reduction in percentage of males was found in traps with either (*E*)-2-hexen-1-ol or Vn added to the pheromone blend (Table 2).

DISCUSSION

Antennae of *I. typographus* of both sexes showed strong EAD responses to 1-hexanol, (*Z*)-3-hexen-1-ol, and (*E*)-2-hexen-1-ol. Weak responses were found to (*E*)-3-hexen-1-ol, (*Z*)-2-hexen-1-ol, and linalool, while no responses were detected to the aldehydes and the acetate. It is possible that *I. typographus* has developed the capability to recognize green leaf alcohols, which are commonly emitted from leaves of nonhost trees, *B. pendula*, *B. pubescens*, *P. tremula*, and *S. nigra* (Byers et al., 1998; Zhang et al., 1999). A similar antennal response pattern has been found for *T. piniperda* and *T. minor* in Europe, but responses seemed to be less sensitive than in *I. typographus* (Zhang et al., unpublished data). In *D. ponderosae*, Wilson et al. (1996) also found antennal responses to the six-carbon green leaf alcohols, but not to the aldehydes. For other bark beetles species, no data are available yet.

The first walking bioassay study by Schlyter et al. (1995) on *I. typographus* and *I. duplicatus* revealed an inhibitory effect by a blend of six GLVs including linalool and a strong synergistic effect by a combination of the GLV blend and Vn. Their attempt to separate the activity of the GLVs blend into three fractions, alcohols, aldehydes and linalool, was less successful. However, our walking bioassay on *I. typographus* indicated no inhibition by a mixture of two aldehydes and the acetate. In contrast, the blend of the three most EAD-active alcohols (3OH-A) significantly inhibited response to the pheromone source, which was similar to the level caused by Vn or the mixture of all nine GC-EAD tested compounds. These three alcohols (3OH-A) were inhibitory not only in a blend but also individually. The additive or synergistic inhibitory effects between the two groups of GLV alcohols (i.e., 3OH-A and 3OH-B), and between Vn and 3OH-A seemed to be significant in the laboratory, but could not be shown in the field assay.

In the field trapping experiments, 3OH-A strongly reduced the trap catches and the proportion of males captured both as a blend and as individual components. The percentage of males captured in the trap baited with pheromone alone in the individual GLV test was higher (37%) than that in the group GLV test (29.6%), which was probably due to the different trapping mechanisms of pipe traps versus funnel traps. This difference is even more pronounced, as the percentage of males normally falls during the flight-season (Lindelöw and Weslien, 1986). However, the blend of three GC-EAD less-active alcohols (3OH-B) that showed little effect in the walking bioassay, did significantly interrupt attrac-

tion in field trapping experiment equal to the level of the negative control, Vn. No effects on trap catches were found when the single components of 3OH-B were tested. Consistent with our GC-EAD and walking bioassay results, neither individual nor the blend of the two aldehydes plus (Z)-3-hexenyl acetate interrupted trap catches. The combination of Vn and 3OH-A had the lowest trap catches, which were obviously lower than that caused by Vn, but not significantly different from those of 3OH-A alone. This is probably due to the quite strong inhibitory effect caused by 3OH-A alone at the current dose. The synergistic effects, if any, might be more relevant if the dose of 3OH-A is reduced. Surprisingly, the blends of all six green leaf alcohols and all nine EAD tested compounds resulted in less disruptive effects on the trap catch than 3OH-A did. On the other hand, the inhibitory effect of the blend of 3OH-A (85% reduction) was higher than that of the single GLV alcohols, 1-hexanol, (Z)-3-hexen-1-ol, and (*E*)-2-hexen-1-ol (41–50%), which might indicate an additive or dose-dependent effect of the individual components. A similar additive effect was previously suggested for *G. sulcatus* by Deglow and Borden (1998a) and for *T. piniperda* by Poland and Haack (2000).

Our results on *I. typographus* are quite similar to those for *D. ponderosae* (Wilson et al., 1996), *T. piniperda* (Poland and Haack, 2000; Zhang et al., unpublished data) and *T. minor* (Zhang et al., unpublished data). None of the GLV aldehydes and the acetate tested was disruptive for these species, while all of the GLV alcohols were active (Wilson et al., 1996; Poland and Haack, 2000; Zhang et al., unpublished data). In *P. chalcographus*, a 60–70% reduction of trap catch was achieved when either 1-hexanol or (Z)-3-hexen-1-ol was added to the pheromone traps (Byers et al., 1998). In North America, the pheromone- or kairomone-positive responses of over 10 species of conifer-attacking scolytids are now known to be disrupted by GLVs (Deglow and Borden, 1998a, and references therein; Poland and Haack, 2000). The most disruptive individual GLV components and blends vary among the different scolytid species. The response diversity to GLVs by conifer bark beetle species might reflect differences in the odor characteristics of their particular habitat and ecosystems (Poland et al., 1998). However, in two coniferophagous ambrosia beetles, *Tryp. lineatum* and *G. sulcatus*, GLV aldehydes, hexanal, and (*E*)-2-hexenal enhanced the response to their pheromones (Borden et al., 1997; Deglow and Borden, 1998a). A similar enhancement was also found in *I. duplicatus* in China when a blend of hexanal, (*E*)-2-hexenal, and (Z)-3-hexenyl acetate was added to the pheromone trap (Zhang and Schlyter, unpublished data).

GLVs are one of the major volatile groups from intact leaves in situ of European birches (*B. pendula* and *B. pubescens*), aspen (*P. tremula*), and alder (*S. nigra*), which commonly occur in the natural habitats of *I. typographus* (Byers et al., 1998; Zhang et al., 1999). All the GLVs tested in the present study, except (*E*)-3-hexen-1-ol and (Z)-2-hexen-1-ol, are released from leaves of the above-men-

tioned nonhost deciduous tree species, with different relative abundances depending on the species (Zhang et al., 1999). Surprisingly, the most dominant component of the GLVs emitted from the leaves of these nonhost trees, (Z)-3-hexenyl acetate (Zhang et al., 1999), has not shown any electrophysiological and behavioral activities in any conifer-infesting bark beetle species tested. However, all three GLV alcohols (3OH-A) that exist and are released from these European nonhost leaves are electrophysiologically and biologically active in *I. typographus*. 1-Hexanol and (Z)-3-hexen-1-ol were recently found from the fresh bark of *B. pendula*, *B. pubescens*, and *P. tremula* in Europe (Zhang et al., 2000), and 1-hexanol was also identified from bark of several North America nonhost angiosperm species (Borden et al., 1998). 1-Hexanol was also detected in trace amounts from the female flowers of *Picea abies* and *Pinus sylvestris*, but not from twigs with needles (Borg-Karson et al., 1985). However, none of these antennally active GLVs were found from aeration samples of fresh bark and branches with needles of the host tree *P. abies* (Zhang et al., unpublished results).

It would be beneficial for conifer bark beetles to be able to recognize and avoid a general volatile signal that is commonly emitted from a wide variety of nonhost deciduous tree species rather than recognizing precise species-specific volatiles for each nonhost species, as discussed by Poland et al. (1998). In this way, several species of nonhost trees with partially overlapping blends of common volatile compounds could be perceived and avoided during host selection (Borden et al., 1998; Poland et al., 1998). The active GLVs from nonhost stands or trees might act as negative signals at the habitat level for conifer-attacking bark beetles when they are seeking hosts during flight (Schlyter and Birgersson, 1999). On the other hand, some specific compounds found in the most prevalent nonhost species (e.g., volatiles from bark of European birches and aspen) could be important as negative signals for close-range host selection and host acceptance after landing (Zhang et al., 2000). The combination of active GLVs, nonhost-specific compounds, and the well-known inhibitor Vn may have great potential in forest protection against conifer-attacking bark beetles.

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GUSTATORY RESPONSE BY THE HYMENOPTERAN PARASITOID *Cotesia glomerata* TO A RANGE OF NECTAR AND HONEYDEW SUGARS

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Abstract—The feeding response of food-deprived *Cotesia glomerata* to solutions of 14 naturally occurring sugars was determined. Glucose, fructose, sucrose, maltose, erlose, melezitose, trehalose, and stachyose all elicited a feeding response. The sugars differed, however, with respect to the lowest concentration at which they were accepted (acceptance threshold). The parasitoids showed no feeding response when presented with 2 M solutions of galactose, mannose, rhamnose, lactose, raffinose, and melibiose. Sugars from the latter group did not show a deterrent effect when offered to water-deprived parasitoids. When mannose, rhamnose, melibiose, or raffinose were combined with low molar solutions of either fructose or sucrose, sucrose acceptance was affected by mannose and raffinose, whereas no negative interactions were found in mixtures with fructose. Compared to acceptance thresholds reported in other insect systems, the responses of *C. glomerata* differ considerably with respect to both the range of saccharides accepted as well as the acceptance thresholds. The novel finding that the parasitoid accepts a number of sugars that fail to elicit a feeding response in its herbivorous hosts is of particular interest to the use of (selective) food supplements in biological control programs.

Key Words—*Cotesia glomerata*, Hymenoptera, sugar, taste, nectar, honeydew, acceptance threshold, sugar receptor, selective, food supplement.

INTRODUCTION

Most (if not all) adult parasitoids will feed on sugar sources (Jervis et al., 1993). Even those species that occasionally feed on the hemolymph of their hosts require saccharides as the main source of energy (Jervis and Kidd, 1986). The

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large number of parasitoid species that do not engage in host feeding are entirely dependent on nectar or honeydew for their nutrition.

Sugar feeding can have strong effects on parasitoid fitness parameters. Several laboratory studies show that sugar can increase parasitoid longevity by factors of up to 20 (Dyer and Landis, 1996; Idoine and Ferro, 1988; Laetemia et al., 1995; Syme, 1975; Wäckers and Swaans, 1993; Zoebelein, 1955). Sugar feeding can also benefit a parasitoid's fecundity, either through a positive effect on the rate of egg maturation, through an increase in reproductive life-span, or both (Laetemia et al., 1995; Olson and Andow, 1998; Syme, 1975; Zoebelein, 1955). Finally, a parasitoid's nutritional state can have a significant impact on its searching behavior. Telenga (1958) and van Emden (1962) found that parasitoids are more active in habitats in which flowers are in bloom than in nearby habitats without flowers. Wäckers (1994) and Takasu and Lewis (1995) demonstrated that sugar deprivation reduces host searching efficiency, partly due to a general reduction in activity and partly to a shift from host searching to food searching.

Under field conditions, parasitoids may obtain saccharides from a broad spectrum of sources. Parasitoid species have been reported to feed on various types of floral nectar (Idris and Grafius, 1997; Jervis et al., 1993; Kevan, 1973), as well as extrafloral nectar (Bugg et al., 1989; Koptur, 1994) and honeydew (Idoine and Ferro, 1988; Zoebelein, 1955). The sugar composition of these food sources can be highly variable (Baker and Baker, 1983a; Bentley, 1977; Kloft et al., 1985). Even though sucrose, and its hexose components fructose and glucose, are the predominant saccharides in most nectars and honeydew, other saccharides can occur as well, sometimes in significant concentrations (Baker and Baker, 1983b; Bentley, 1977; Kloft et al., 1985). Little information is available regarding the suitability of individual saccharides as food sources for the ecologically and economically important group of Hymenopteran parasitoids.

The suitability of sugars is determined both at the sensory level (gustatory stimulation) and at the physiological level (digestion and energy convertibility). The latter aspect is addressed separately (Wäckers, in preparation). The present paper concentrates on aspects of parasitoid taste perception. *Cotesia glomerata* (Hymenoptera: Braconidae) was chosen for the experiments, based on the fact that this parasitoid refrains from feeding on host hemolymph or pollen (Wäckers, personal observations). It therefore represents the large group of parasitoids whose diet is restricted to nectar and honeydew. *C. glomerata* visits various nectar sources in the field (Hirose, 1966; Wäckers, unpublished), and sugar feeding can have a dramatic impact on the longevity of *Cotesia* spp. (Wäckers and Swaans, 1993; Wäckers, in preparation).

To determine the gustatory response of *Cotesia glomerata* with respect to a range of naturally occurring sugars, the parasitoid's reaction to 14 saccharides was tested. With the exception of lactose, all of these sugars have been reported to occur in nectar or honeydew (Baker and Baker, 1983b; Bentley, 1977; Kloft

et al., 1985). The fact that most of the saccharides tested had also been included in similar experiments with honeybees and ants (von Frisch, 1934; Schmidt, 1938) allowed for comparisons of gustatory responses among these Hymenopteran species.

The lowest concentration at which *C. glomerata* would accept the solution as a food source (acceptance threshold) was determined for all 14 saccharides. Since a lack of feeding response can either indicate lack of phagostimulation, phagodeterrency, or a combination of both (Stoffolano, 1995), those saccharides that were not accepted at the highest (2 M) concentration were used in further experiments to test for feeding deterrency. In addition mannose, rhamnose, melibiose, and raffinose were combined with low molar solutions of either fructose or sucrose to test for possible negative interactions between these sugars (Dethier et al., 1956).

METHODS AND MATERIALS

Insects. Cotesia glomerata were reared on *Pieris brassicae* fed with Brussels sprouts plants [*Brassicae oleracea* (L.) var. *Gemmifera*] at 21°C, 60% relative humidity, and a 16L : 8D photoperiod. Parasitoid coccoons were collected and transferred to a climate chamber (15°C, 75% relative humidity, 16L : 8D). Upon emergence, groups of 20–30 parasitoids of both sexes were transferred to polypropylene cages (30 × 30 × 30 cm) and provided with water only (food-deprived parasitoids), or no food and no water (water-deprived parasitoids). Two-day-old (presumably mated) females were used in the experiments. This age was chosen because the life-span of food-deprived *C. glomerata*, under the given conditions, is two to three days (Wäckers, unpublished). Any parasitoids showing visible signs of energy deprivation at the time of the experiment were discarded.

Acceptance Threshold. The acceptance threshold was determined by presenting food-deprived parasitoids with solutions of one of the 14 sugars listed in Table 1. To ensure that test insects were water-satiated at the time of the experiment, parasitoids were placed individually in a small glass vial containing wet filter paper for a period of 30 min prior to the experiments. Subsequently, parasitoids were transferred to a second vial, the bottom of which contained a 5-μl droplet of a sugar solution. The test vial was placed upside down on a wet filter paper to avoid a concentration increase in the test solution due to evaporation. As soon as the parasitoid's tendency to walk upwards had brought it in contact with the sugar solution, its feeding response was recorded. The reaction was either scored as acceptance (feeding for more than 5 sec) or rejection (less than 5 sec of contact with the solution).

To determine the lowest concentration at which sugars evoked a feeding response in the food-deprived parasitoids (acceptance threshold), sugar concen-

TABLE 1. SOURCE AND PURITY OF SUGARS USED IN EXPERIMENTS

		Source	Purity (%)
Monosaccharides			
D-(+)-Glucose		Fluka	>99
D-(−)-Fructose		Merck	>99
D-(+)-Galactose		Fluka	>99
D-(+)-Mannose		Aldrich	>99
L-(+)-Rhamnose monohydrate		Fluka	>99
Disaccharides			
D-(+)-Sucrose	β-D-fructofuranosyl-(2 ↔ 1)-α-D-glucopyranoside	Fluka	>99
D-(+)-Trehalose	α-D-glucopyranosyl-(1 ↔ 1)-α-D-glucopyranoside	Fluka	>99
D-(+)-Maltose monohydrate	α-D-glucopyranosyl-(1 → 4)-D-glucose	Fluka	>99
D-(+)-Melibiose monohydrate	α-D-galactopyranosyl-(1 → 6)-D-glucose	Aldrich	>99
D-(+)-Lactose monohydrate	β-D-galactopyranosyl-(1 → 4)-D-glucopyranose	Fluka	>99
Trisaccharides			
D-(+)-Raffinose pentahydrate	β-D-fructofuranosyl-(2 ↔ 1)-α-D-galactopyranosyl-(1 → 6)-α-D-glucopyranoside	Fluka	>99
D-(+)-Melezitose monohydrate	α-D-glucopyranosyl-(1 → 3)-β-D-fructofuranosyl-(2 ↔ 1)-α-D-glucopyranoside	Fluka	>99
Erlose monohydrate	β-D-fructofuranosyl-(2 ↔ 1)-α-D-glucopyranosyl-(1 → 4)-α-D-glucopyranoside	Senn	98.9
Tetrasaccharide			
Stachyose tetrahydrate	β-D-fructofuranosyl-(2 ↔ 1)-α-D-galactopyranosyl-(1 → 6)-α-D-galactopyranosyl-(1 → 6)-α-D-glucopyranoside	Aldrich	98

tration series were presented to separate parasitoid cohorts. At the start of each series, the sugar was offered in a 2 M concentration to a total of 25 individual parasitoids. This concentration was chosen as it represents the high end of sugar concentrations found in floral nectar and honeydew (Baker and Baker, 1983a; Kloft et al., 1985). When at least one individual accepted the solution, a new group of females was tested at half the concentration (1 M). This procedure was repeated with decreasing concentrations of the sugar solution (1/2, 1/4, 1/8, 1/16, 1/32, 1/64, and 1/128 M). The experiment was terminated at the concentration at which none of the 25 parasitoids responded.

Feeding Deterrency of Individual Sugars. Those saccharides that failed to

evoke a feeding response at a 2 M concentration (i.e., raffinose, melibiose, galactose, mannose, lactose, and rhamnose) were subsequently examined with respect to possible feeding deterrency. For this purpose, the liquid uptake by water-deprived parasitoids was determined when presented with either distilled water or a solution containing 2 M of the nonstimulatory sugars. Consumption was determined by weighing the individual parasitoids on a precision scale (Mettler MT5; ± 2 μg) immediately before and after exposure to the liquid. Each solution was offered to separate cohorts of 20 parasitoids. As a further control an additional group of parasitoids was given a 2 M sucrose solution.

Feeding Deterrency of Sugar Mixtures. Mannose, rhamnose, melibiose, and raffinose were also examined with respect to possible feeding inhibitory effects when combined with the stimulatory sugars sucrose or fructose. In this experiment, food-deprived parasitoids were presented with a solution containing 2 M of the nonstimulatory sugar together with 1/8 M of either sucrose or fructose. The 1/8 M concentration was chosen as this was the lowest concentration at which these sugars were still predominantly accepted. This concentration could consequently be expected to be sensitive to possible feeding inhibition by the added sugar. Twenty parasitoids were tested with each sugar combination. Separate groups were tested with 1/8 M sucrose or fructose as a control. Feeding inhibition was concluded if significantly fewer parasitoids fed from the sugar combination than from the 1/8 M solution.

RESULTS

Acceptance Threshold. The 14 saccharides tested differed considerably with respect to their phagostimulatory effect on *C. glomerata.* Only eight sugars evoked any kind of feeding response in the food-deprived parasitoids (Figure 1). Of these, parasitoids were most sensitive to fructose. Half the parasitoids still responded to this sugar at 1/64 M, a concentration at which none of the other sugars was still stimulatory. *C. glomerata*'s threshold of acceptance for erlose was 1/32 M, for glucose and sucrose 1/16 M, for melezitose 1/4 M, for maltose and trehalose 1/2 M, and for stachyose 1 M.

Aside from the differences in acceptance threshold, the shape of *C. glomerata*'s response curve varied among the sugars tested (Figure 1). While the response to sugars like erlose and glucose declined slowly over a wide range of concentrations, the response to sucrose remained high up to a concentration of 1/8 M, but declined sharply in response to further dilutions. The response curve in the case of melezitose was unusual as it remained constant at a rather low (50–60%) level over the concentration range of 2 M–1/2 M.

Six sugars (galactose, lactose, mannose, melibiose, raffinose, and rhamnose) failed to evoke any feeding response in food-deprived parasitoids even in a 2 M

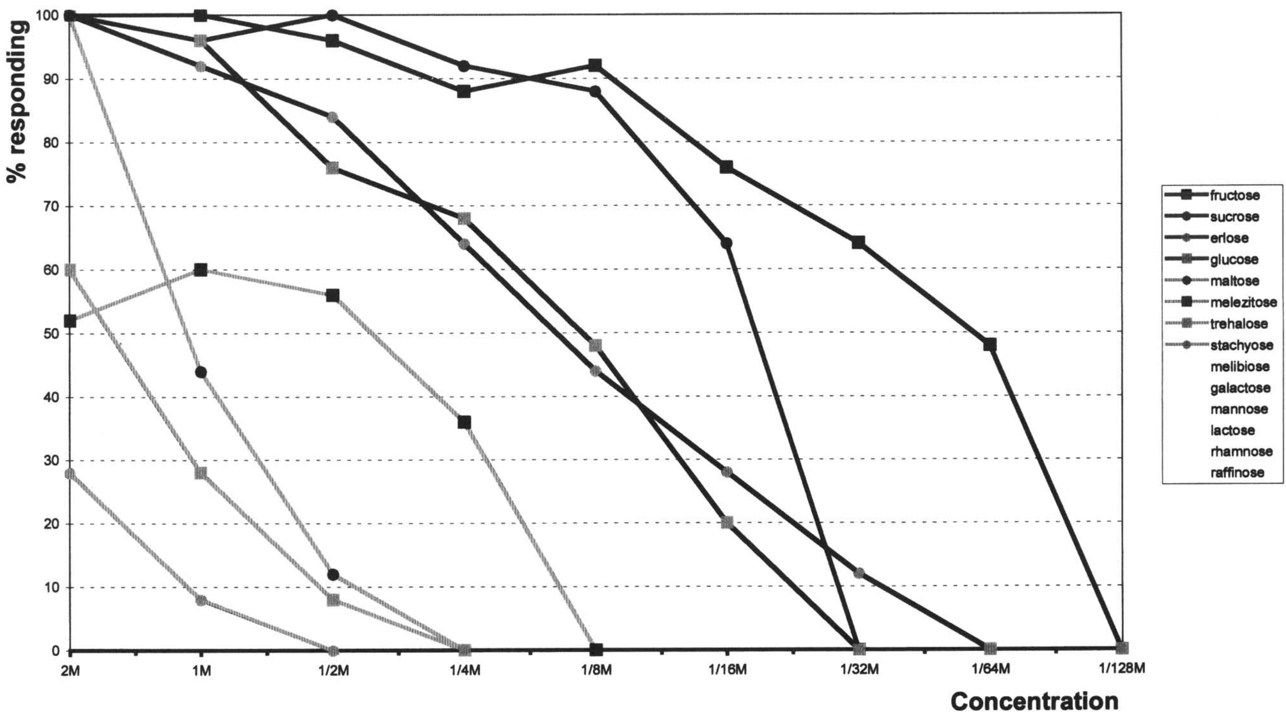

FIG. 1. Percentage of food-deprived *Cotesia glomerata* responding to sugar solutions of various concentrations. Individual cohorts of 25 parasitoids were used for each sugar and each concentration. Two molar solutions of melibiose, galactose, mannose, lactose, rhamnose and raffinose failed to elicit a feeding response.

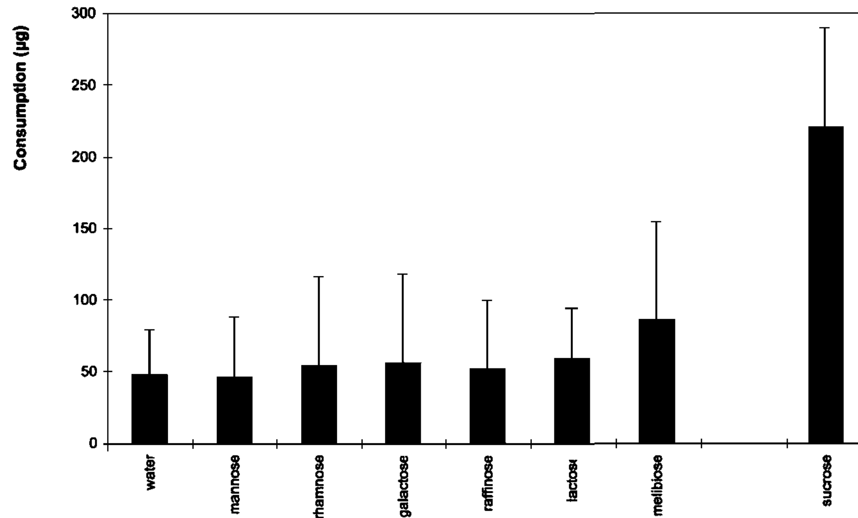

FIG. 2. Average liquid uptake (+SD) by 20 food- and water-deprived parasitoids when presented with a solution containing 2 M of one of the nonstimulatory sugars. Separate cohorts were offered distilled water or sucrose (2 M) as controls.

concentration. Consequently, no acceptance threshold could be established for these sugars.

Feeding Deterrency of Individual Sugars. Parasitoids that had been deprived of both water and food readily accepted the solutions of each of the nonstimulatory sugars. None of the sugars reduced the liquid intake in comparison to the water control (Figure 2). A significant increase in liquid consumption over the control was only found in the case of the sucrose solution (ANOVA, $P < 0.001$).

Feeding Deterrency of Sugar Mixtures. Mannose and raffinose inhibited the parasitoid's feeding response when offered in combination with 1/8 M sucrose (Table 2) but had no inhibiting effect on the acceptance of fructose. Melibiose and rhamnose did not inhibit the feeding response to either sucrose or fructose.

DISCUSSION

Insects possess gustatory receptors on a wide range of structures (Chapman, 1995). In the case of Hymenoptera (*Apis mellifera*), sugar receptors have been reported on tarsi (Minnich, 1932; Marshall, 1935; Whitehead and Larsen, 1976), antennae (Marshall, 1935; Bitterman et al., 1983), and mouthparts (Whitehead and Larsen, 1976). A feeding response (proboscis extension) can be elicited

TABLE 2. TEST OF FOUR NONSTIMULATORY SUGARS FOR POSSIBLE INHIBITORY EFFECTS ON FEEDING RESPONSE BY *Cotesia glomerata* TO SUCROSE AND FRUCTOSE[a]

Sugar (2 M)	In mixture with 1/8 M sucrose	Control (1/8 M sucrose)	χ^2 test	In mixture with 1/8 M fructose	Control (1/8 M fructose)	χ^2 test
Mannose	7	20	<0.0001	18	20	NS
Melibiose	20	20	NS	18	18	NS
Raffinose	9	19	0.004	20	17	NS
Rhamnose	16	18	NS	20	20	NS

[a]Figures represent the number of parasitoids (out of a total of twenty) showing a feeding response to a mixture of a nonstimulatory sugar (2 M) and a stimulatory sugar (1/8 M). As a control, separate cohorts were given 1/8 M of the stimulatory sugar only.

by stimulation of any of these receptors (Minnich, 1932; Kuwabara, 1957; Bitterman et al., 1983). Food-deprived *Cotesia* frequently contact the substrate with their mouthparts, irrespective of tarsal stimulation (Wäckers, 1994). In the present experiments, parasitoids typically sampled the droplet with their mouthparts before accepting or rejecting the sugar solution, making it likely that the acceptance thresholds can be attributed to mouthpart receptors. However, since tarsal and antennal contact could not be excluded, the involvement of other taste receptors cannot be ruled out.

C. glomerata responded to eight of the 14 carbohydrates tested. Contact with the other sugars did not elicit feeding. The parasitoids were most sensitive to those sugars (sucrose, fructose, and glucose) which are the primary components of most nectars and honeydews (Baker and Baker, 1983a; Kloft et al., 1985). The acceptance threshold for these sugars was considerably lower than the concentrations (10–50% w/v) at which they naturally occur (Baker and Baker, 1983a; Kloft et al., 1985). Therefore, this sensitivity spectrum likely allows *C. glomerata* to detect all of its potential food sources.

While a feeding response demonstrates perception, lack of perception cannot be deduced from lack of feeding initiation. When a saccharide fails to elicit a feeding response in a food-deprived individual, this either indicates that this compound does not stimulate the sugar receptor or that it produces a deterrent response (phagodeterrency stronger than phagostimulation) (Dethier, 1976). The experiments with the water-deprived parasitoids show that the lack of response by water-satiated individuals is probably not based on deterrency, therefore indicating that these sugars fail to adequately stimulate the sugar receptors.

The contact chemoreceptor sensillae of insects generally contain several chemosensitive neurons. These include the so-called sugar cell, water cell, and the (deterrent) salt cell (Chapman, 1995). Whether a receptor neuron is suitable for the perception of a particular sugar depends on the type of receptor proteins

as well as the molecular configuration of the sugar. This realization has led to attempts to elucidate the molecular basis of receptor specificity by correlating carbohydrate configuration and stimulating effect.

Following the pioneering work by Dethier (overview in Dethier, 1976), detailed structural requirements for receptor affinity have been elucidated in calyptrate flies. At least two separate sugar receptor sites have been described in *Phormia regina* and *Boettcherisca peregrina*, the so-called pyranose site, and the furanose site (Evans, 1963; Shimada et al., 1974; Shimada and Isono, 1978). It is generally assumed that the pyranose site binds primarily with sugar molecules containing equatorial hydroxyl groups on C-2, C-3, and C-4 such as glucose, maltose, sucrose, and trehalose (Evans, 1963; Morita and Shiraishi, 1985). In *B. peregrina* the furanose site is thought to be the primary site for fructose, galactose, and mannose perception (Shimada et al., 1974; Shimada and Isono, 1978). The specific configuration of the furanose site, however, is less well understood.

The fact that insect species often differ considerably with respect to both the range of saccharides accepted, as well as their acceptance thresholds, indicates that the molecular basis of sugar perception as it has been revealed for species like *P. regina* and *B. peregrina* does not necessarily translate to other systems. The presented data from *Cotesia glomerata* do not contradict the existence of a pyranose site as described in calyptrate flies. In this parasitoid as well, the configuration of the C-2, C-3, and C-4 hydroxyl groups seem to be important for acceptance. Pyranose-type mono- and disaccharides (glucose, maltose, sucrose, and trehalose) with equatorial hydroxyl groups were accepted, while mannose and rhamnose with axial hydroxyl groups at C-2 and C-4, believed to interfere with the pyranose site, did not elicit feeding.

The findings presented do not, however, support the existence of a blowfly-type furanose site in *C. glomerata.* Even though the parasitoid showed highest sensitivity to fructose, it failed to respond to galactose and mannose, the other sugars thought to bind to the furanose site in calyptrate flies. As a matter of fact, none of the carbohydrates containing a galactopyranosyl group was phagostimulatory, and both monosaccharides containing mannohexose (mannose and rhamnose) failed to elicit a response. Furthermore, inhibition of the fructose response by mannose and raffinose, a phenomenon reported for the blowfly and crucial to the description of the furanose site (Dethier et al., 1956; Wieczorek and Wolff, 1989), did not occur in *C. glomerata.* These findings indicate that the receptor configuration in *C. glomerata* differs from the configuration proposed for calyptrate flies.

Data available from comparable feeding studies with the honeybee (von Frisch, 1934) and the ant species *Myrmica rubra*, *M. rubida*, and *Lasius niger* (Schmidt, 1938) allow us to put the acceptance thresholds of *Cotesia glomerata* in perspective to those reported for other Hymenoptera (Table 3). *C. glomerata* largely matches the honeybee with respect to the spectrum of sugars that

evoke a feeding response. However, some interesting quantitative differences in acceptance thresholds are apparent between the two species. Honeybees show the lowest acceptance threshold for sucrose (1/16 M), and are relatively less responsive to hexose sugars fructose and glucose (1/4 M each). While *C. glomerata* is equally responsive to sucrose (1/16 M), it shows a considerably lower acceptance threshold for the hexose sugars glucose (1/16 M) and especially fructose (1/64 M). These differences in responsiveness correlate with the sugar composition of the nectar sources exploited by these species. Long-tongued bees, such as *A. mellifera*, usually visit flowers with a longer corrola and sucrose-rich nectars (Baker and Baker, 1983b). *C. glomerata*, like the majority of parasitoids (see Jervis (1998) for exceptions), has unspecialized mouthparts. These parasitoids are consequently restricted to exploiting exposed nectaries (Kevan and Baker, 1983; Wäckers et al., 1996), the nectar of which is often dominated by glucose and fructose (Percival, 1961; Baker and Baker, 1983a; Bentley, 1983). It would be interesting to determine whether parasitoids whose specialized mouthparts (Jervis, 1998) allow access to the sucrose-rich honeybee-pollinated flowers are more sucrose sensitive than *C. glomerata.*

The ant species tested by (Schmidt, 1938) differ considerably from both *C. glomerata* and *A. millifera* in their sugar responses (Table 3). They correspond with the honeybee in being more sensitive to sucrose than to fructose and glucose. Overall, however, their threshold of response is approximately 10-fold more sensitive than the response levels in *C. glomerata* and *A. mellifera.* It is also notable that ants respond to raffinose, a sugar that failed to elicit any response in either *C. glomerata* or *A. mellifera.* This particular sensitivity might be an adaptation that allows ants to locate colonies of honeydew-producing Sternorrhynchae, which often represent the main source of carbohydrates for ant species (Tobin, 1994). The high overall sugar sensitivity could enable scouting ants to detect even low concentrations of washed down honeydew, whereas raffinose, being a common honeydew component (Völkl et al., 1999) can be used as a reliable indicator of sap feeders.

While Hymenopteran species show substantial variation with respect to the range of saccharides perceived and the concentration threshold of acceptance, the differences among insect orders can be even more marked (Table 3). When natural or artificial food sources are used in agroecosystems to increase the effectiveness of predators or parasitoids (e.g., Powell, 1986; Hagen, 1986; Bugg et al., 1991; Canas and O'Neil, 1998), these differences in acceptance thresholds can have important applied implications. The use of food supplements to augment biological control entails the potential drawback that pest organisms may also benefit from the food source. Nectar feeding can increase herbivore longevity (e.g., Binder, 1996), as well as the number and size of matured eggs (Leahy and Andow, 1994; McEwen and Liber, 1995). When adult herbivores are attracted or retained by nectar, this can lead to a considerable increase in herbivory lev-

TABLE 3. COMPARISON OF SUGAR ACCEPTANCE THRESHOLDS IN *Cotesia glomerata* WITH THOSE PREVIOUSLY REPORTED FOR OTHER INSECTS[a] (VALUES GIVEN IN MOL)

	Hymenoptera (min. threshold)					Diptera (ED_{50})		Lepidoptera (ED_{50})	Orthoptera (min. threshold)
	Cotesia glomerata	*Apis mellifera*	*Myrmica rubra*	*Myrmica rubida*	*Lasius niger*	*Phormia regina*	*Culiseta inornata*	*Spodoptera littoralis*	*Locusta migratoria*
Monosaccharides									
Glucose	1/16	1/4	1/50	1/32	1/8	0.13	0.24	0.113	0.125
Fructose	1/64	1/4	1/50	1/32	1/64	0.006	0.2	0.008	0.025
Galactose	NR	2	0.5	NR	NR	0.5		NR	0.025
Mannose	NR	NR	0.25	NR	NR	7.6		NR	NR
Rhamnose	NR	NR	1	NR	NR			0.092	NR
Disaccharides									
Sucrose	1/16	1/16	1/150	1/400	1/200	0.01	0.062	0.009	0.005
Trehalose	1/2	1/4					0.15		0.125
Maltose	1/2	1/8	1/100	1/100	1/100	0.004	0.17	1.08	0.025
Melibiose	NR	NR	NR	NR		NR	0.45	NR	0.005
Lactose	NR	NR	NR	NR	NR		0.4	NR	0.025
Trisaccharides									
Raffinose	NR	NR	1/400	1/100	1/400	0.2	0.15	0.124	0.005
Melezitose	1/4	1/8	1/200	1/64	1/100	0.064	0.011	0.294	0.005
Erlose	1/32								
Tetrasaccharide									
Stachyose	1								
Study	This one	von Frisch (1934)	Schmidt (1938)	Schmidt (1938)	Schmidt (1938)	Hassett et al. (1950)	Schmidt and Friend (1991)	Salama et al. (1984)	Cook (1977)

[a]NR = no response; open cells indicate that the particular combination was not tested.

els (Hagen, 1986; McEwen and Liber, 1995). Knowledge on sugar perception by both natural enemies and pest insects could help us develop strategies to avert these negative effects. When comparing the range of sugars accepted by *Cotesia glomerata* with those acceptable to its hosts *Pieris rapae* and *P. brassicae*, some clear differences emerge. While the parasitoids readily accept a rather broad range of sugars, the adult herbivores fail to show a consistent response to any sugars other than sucrose and fructose (Kusano and Sato, 1980; Romeis and Wäckers, in preparation). This is the first report to demonstrate that parasitoids exhibit gustatory responses to sugars that are not accepted by their herbivorous hosts. This specificity has important implications for the use of food supplements in biological control programs, as it means that food supplements can be selectively attuned to the palate of the antagonist, without being acceptable to the herbivore. In the tritrophic system of *Cruciferae*, *Pieris* spp., and *Cotesia glomerata*, the common sugar glucose could provide such selectivity.

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