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AN ALLELOCHEMICAL ELICITS ARRESTMENT IN *Apanteles kariyai* IN FECES OF NONHOST LARVAE *Acantholeucania loreyi*

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Abstract—Females of the larval parasitoid *Apanteles kariyai* (Watanabe) (Hymenoptera: Braconidae) are arrested on fecal pellets of *Acantholeucania loreyi* (Duponchel) (Lepidoptera: Noctuidae) larvae. Upon subsequent antennal contact with an *A. loreyi* larva, females sting it with their ovipositor. However, such stinging did not result in any offspring. The allelochemical involved in feces has been identified and is identical to a kairomone of the host *Pseudaletia separata* (Lepidoptera: Noctuidae). In contrast to *A. loreyi*, *P. separata* is a suitable host for *A. kariyai*, and oviposition in *P. separata* results in offspring production. The allelochemical mediating the interaction between *A. loreyi* and *A. kariyai* is discussed in the context of current allelochemical terminology.

Key Words—*Apanteles kariyai*, Hymenoptera, Braconidae, *Acantholeucania loreyi*, *Pseudaletia separata*, Lepidoptera, Noctuidae, 2,5-dialkyltetrahydrofuran, arrestant, allelochemical, antimone, kairomone.

INTRODUCTION

Apanteles kariyai is one of the dominant larval parasitoids of the common armyworm, *Pseudaletia separata* in Japan (Hirai, 1984). This wasp searches for and attacks the host larvae actively during daytime while the nocturnal host often hides on the ground or in the sheath of host plants (Sato et al., 1983; Kanda, 1988).

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The host-finding behavior of this wasp has been studied in the laboratory by Sato et al. (1983) and by Takabayashi et al. (1985). When an *A. kariyai* female contacts feces, feeding traces, or exuviae of host larvae with her antennae, she is immediately arrested and starts searching around these materials. Upon subsequent antennal contact with a *P. separata* larva, she immediately inserts her ovipositor and deposits eggs inside the larva. Contact kairomones that arrest *A. kariyai* have been isolated from the feces, feeding traces, and exuviae of *P. separata* larvae (Takabayashi et al., 1985). The chemical structures of these arrestants were identical for each of the three sources and were 2,5-dialkyltetrahydrofuran homologs (Takabayashi and Takahashi, 1986a). The chemical structure–arrestment activity relationship of this compound was tested; the presence of alkyl groups at least longer than octyl at the 2 and 5 positions of the tetrahydrofuran ring induced the arrestment response to *A. kariyai* (Takabayashi and Takahashi, 1986b).

Pseudaletia separata is polyphagous and its larvae feed on leaves of many Gramineae species such as rice, wheat, millet, sugarcane, foxtail, radish, Chinese cabbage, cotton, buckwheat, etc. On the other hand, *A. kariyai* is a monophagous parasitoid of *P. separata* larvae (Yasumatu and Watanabe, 1964). In the host habitat, therefore, there will probably be other nonhost noctuid larvae that feed on the same plant species; *Acantholeucania loreyi* is one of these nonhost noctuid larvae.

In this paper, we report the response of *Apanteles kariyai* upon contact with fecal pellets from *Acantholeucania loreyi* larvae and the subsequent response upon contact with fifth-instar larvae. The allelochemical involved in feces was isolated and compared with that of *P. separata*. The effect of this allelochemical on the host-searching behavior of *A. kariyai* is discussed in the context of current allelochemical terminology.

METHODS AND MATERIALS

Insects. Pseudaletia separata was introduced in the laboratory from a stock culture at Takeda Chemical Industries Ltd. *Acantholeucania loreyi* were from a stock culture at the laboratory of Zoology, Faculty of Science, Kyoto University. Both species were reared on corn leaves (25 ± 2°C, 17:7 light–dark, 50–70% relative humidity), *P. separata* for more than five years and *A. loreyi* for one year. About 50 larvae of each species were reared in a plastic container (20 × 27 × 10 cm). *Apanteles kariyai* was collected in Kanoya, Kagoshima, Japan, and has been reared for more than five years in the laboratory. Either fourth- or fifth-instar *P. separata* were offered to adult parasitoids for oviposition. Newly emerged wasps were placed in a test tube (22 mm diameter, 200 mm long) with a small piece of paper towel moistened with a 50% aqueous

honey solution as food. Mating generally occurred immediately upon emergence of the female. One day after emergence, females were separated from males and kept in a climate box (10°C) in darkness for at least five days prior to the experiment. Subsequently, they were transferred to a climate room (25 ± 2°C, 50–70% relative humidity), where they were kept at least 1 hr before the experiment.

Experimental Conditions. Experiments were carried out under fluorescent light at 25 ± 2°C, and 50–70% relative humidity. Female *A. kariyai* were introduced to the experimental area individually. None of the *A. kariyai* females used in this study had previous oviposition experience.

Experiment A: Response of A. kariyai *to Fecal Pellet of* A. loreyi *Larvae.* A fresh fecal pellet from fifth-instar *A. loreyi* was put in the center of a filter paper (19 cm diameter) in a Petri dish (19 cm diameter, 3 cm height), which served as the observation arena. The antennal contact response of *A. kariyai* to a fecal pellet was observed directly. Observation was terminated 10 min after her first contact with a fecal pellet.

Experiment B: Response of A. kariyai *to* A. loreyi *Larvae.* The arena described in experiment A was used in this experiment; a fifth-instar *A. loreyi* was offered to the wasp by forceps after the wasp had found a fecal pellet from *A. loreyi* and had begun the arrestment response to it. As a control, a *P. separata* larva was offered to a wasp after the wasp had found a fecal pellet from *P. separata* and had begun the arrestment response to it. Larvae of the two species attacked by the wasp were reared separately for about 10 days (25 ± 2°C, 17:7 light–dark, 50–70% relative humidity), and the number of larvae from which the wasp hatched was counted.

Chemical Analysis. The same extraction and purification methods as used for analysis of the arrestant of *P. separata* larvae were applied in this study (Takabayashi et al., 1985). Feces were collected and soaked in hexane. The extract was chromatographed on a silicic acid column and eluted successively with hexane, benzene, ether, and methanol. The active fraction was detected by means of a bioassay in which a filter paper disk (6 mm diameter) impregnated with test solution was offered to the wasp (Takabayashi et al., 1985). The filter paper disk impregnated with the fraction possessing arrestment activity elicited intense and continuous antennal contact at the wasp's first contact. The active eluate was further purified using high-performance liquid chromatography (HPLC) (column: silicic acid, 8 mm diameter × 300 mm long; detector: Waters R-401 differential refractometer) with 0.2% ether in hexane (flow rate: 1 ml/min).

Instrumental Analysis. Mass spectra were measured with a Hitachi model M-80 spectrometer combined with a gas chromatograph (1% OV-1, 50 cm, 250–300°C, 4°C/min); ionization voltage was 70 eV.

RESULTS

Experiment A: Response of A. kariyai *to Fecal Pellet of* A. loreyi *Larvae.* Upon release, *A. kariyai* walked on the observation arena. However, when *A. kariyai* found the fecal pellet of *A. loreyi*, she intensively contacted it with her antennae and stayed near it, standing still or walking slowly. During antennal contact, *A. kariyai* sometimes left the fecal pellet but returned immediately and made intensive antennal contact again. The wasp repeated this behavior of leaving and returning to the fecal pellet (excursion) several times until she rejected the fecal pellet. We called these sequences of antennating behavior and excursion an arrestment response. All wasps ($N = 20$ for each species) demonstrated the same arrestment response upon first contact with a fecal pellet of either *A. loreyi* or *P. separata* (Table 1).

Experiment B: Response of A. kariyai *to* A. loreyi *Larvae.* When *A. kariyai* found an *A. loreyi* larva during the arrestment response to a fecal pellet of *A. loreyi*, she immediately mounted it and inserted her ovipositor. However, this never resulted in any offspring ($N = 20$) (Table 1). In contrast, when *P. separata* larvae were offered to the wasp, 70% of all ovipositor insertions resulted in offspring production (Table 1).

Chemical and Instrumental Analysis. The fraction possessing arresting activity gave a single peak on HPLC (silicic acid column) with a retention time of 10 min. This retention time is the same as that of previously identified arrestants of *P. separata*, 2,5-dialkyltetrahydrofuran homologs (Takabayashi et al., 1985; Takabayashi and Takahashi, 1986a). By measurement of the gas–liquid chromatography–mass spectrometry (GLC-MS), arrestants of *A. loreyi* were found to be a series of homologs and M^+ of major homologs are 478, 506, 534, which are the same as those of arrestants of *P. separata*. Each homolog in arrestants of *A. loreyi* showed the characteristic daughter ion peaks caused by the α-fission between the ring and alkyl chain group of 2,5-dialkyltetrahydrofuran, which already had been observed in the mass spectra of arrestants of *P. separata* (Takabayashi and Takahashi, 1986a).

Comparison of mass spectra and HPLC retention times of arrestants of *P.*

TABLE 1. RESPONSE OF *Apanteles kariyai* TO *Pseudaletia separata* AND *Acantholeucania loreyi* LARVAE AND THEIR FECAL PELLETS

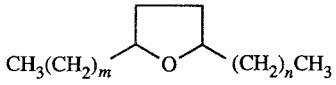
Larvae	No. of larvae	Arresting response to a fecal pellet (%)	Oviposition behavior (%)	Parasitoid egression (%)
P. separata	20	100	100	70
A. loreyi	20	100	100	0

separata, which already had been identified as 2,5-dialkyltetrahydrofuran homologs (Takabayashi and Takahashi, 1986a), with those of *A. loreyi* showed they were identical. From the assignment of characteristic daughter ions in the mass spectrum, each homolog identified in feces of *A. loreyi* contained several structural isomers. The chemical structures of the major homologs in arrestants of *A. loreyi* and *P. separata* are compared in Table 2. Most of the structural isomers in each homolog from two noctuid species are the same, but two new isomers were found in the feces of *A. loreyi*: 2-nonyl-5-eicosyltetrahydrofuran (*m/z* 478) and 2-nonyl-5-tetracosyltetrahydrofuran (*m/z* 534). Isolated arrestants of *A. loreyi* and *P. separata* both elicited the arrestment response to *A. kariyai* at an amount of 10^{-4} μg per filter paper disk and above.

DISCUSSION

The arrestment response of *A. kariyai* to a fecal pellet of *A. loreyi* was characterized by a repetition of stopping or slowed walking with intense antennating response followed by excursion, as also has been observed as the behavioral response of this wasp to a fecal pellet of a host, *P. separata* larva (Takabayashi and Takahashi, 1989). The arrestment response of *A. kariyai* to

TABLE 2. CHEMICAL STRUCTURE OF MAJOR COMPOUNDS OF ARRESTANTS IN FECES OF *Pseudaletia separata* AND *Acantholeucania loreyi* LARVAE



M^+	m^a	n^a	Arrestants isolated from feces of	
			P. separata	*A. loreyi*
478	8	19	–[b]	+
	10	17	+	+
	12	15	+	+
506	10	19	+	+
	12	17	+	+
	14	15	+	+
534	8	23	–	+
	10	21	+	+
	12	19	+	+
	14	17	+	+
	16	15	+	+

[a] Number of methylene in alkyl group of 2,5-dialkyltetrahydrofuran.
[b] – = absence, + = presence.

feces of the host larvae is considered to be one of the important factors for the wasp's host-finding behavior (Sato et al., 1983; Takabayashi and Takahashi, 1989).

The fact that *A. kariyai* demonstrated arrestment response to a fecal pellet of nonhost larvae *A. loreyi* (experiment A) and the ovipositor insertion to *A. loreyi* larva itself (experiment B) indicated that the wasp behaves in the laboratory as if *A. loreyi* is a suitable host. *A. loreyi* and *P. separata* are closely related to each other and feed on leaves of the same Gramineae species in the field. *Apanteles kariyai*, once on a Gramineae species infested by *A. loreyi* larvae in the field, will encounter feces left by the noctuid larvae on the ground, on the leaves, and/or in the sheaths of the plants, and begin the arrestment response to them to search for the host as does this wasp to feces of host larvae on corn plants (Takabayashi, 1986). However, even if *A. kariyai* finds and attacks *A. loreyi* larvae in their habitat after finding feces of *A. loreyi*, no offspring are produced in *A. loreyi* larvae (experiment B). Thus, the response of *A. kariyai* to arrestants of *A. loreyi* involves costs (energy for searching) but no benefits (offspring). Arrestants in feces of *A. loreyi* may be a relic of past interaction between two organisms: emitter and receiver, e.g., *A. loreyi* and *A. kariyai* or their ancestors. It is not demonstrated here whether *A. loreyi* also pays costs as results of production of arrestants for *A. kariyai* and being attacked by *A. kariyai*. However, stinging of *A. kariyai* by *A. loreyi* is at least not adaptive to *A. loreyi*. Further study is necessary on this point.

Because *A. loreyi* larvae (nonhost) occur in the same habitat as *P. separata* larvae and produce an arrestant for *A. kariyai*, but nevertheless are unavailable for offspring production by *A. kariyai*, *A. loreyi* is named here pseudohost to discriminate other nonhosts that have no effects on the host-finding behavior of *A. kariyai*.

There were two new isomers found in arrestants of *A. loreyi* (Table 2). Since the presence of alkyl groups at least longer than octyl (C_8) at the C-2 and C-5 of the tetrahydrofuran ring induced arrestment activity (Takabayashi and Takahashi, 1986b), and these two isomers' alkyl chain lengths are both longer than nonyl (C_9), these two isomers probably possess arrestment activity as well.

An allelochemic is defined as a chemical significant to an organism of a species different from its source, for reasons other than food (Whittaker, 1970). This term is now considered to be switched to "allelochemical" (e.g., Kogan, 1986; Bowers, 1988; Dicke and Sabelis, 1988; Whitman, 1988). Therefore, arrestants of *A. loreyi* can be considered to be allelochemicals. Allelochemicals have been shown to be involved in the following interactions; (1) adaptive to both emitter and receiver (synomone), (2) adaptive to the emitter but not to the receiver (allomone), and (3) adaptive to the receiver but not to the emitter (kairomone) (Nordlund and Lewis, 1976; Nordlund, 1981; Dicke and Sabelis, 1988). However, interactions that are adaptive to neither the emitter nor the

receiver were never previously discussed. From the results of experiment A and B, arrestants of *A. loreyi* evoke in *A. kariyai* an arrestment response that is at least adaptive to neither receiver (*A. kariyai*) nor emitter (*A. loreyi*) as we mentioned above.

Whitman (1988) recently proposed a term "antimone" for the new class of allelochemicals: substances produced or acquired by an organism that, when it contacts an individual of another species in the natural context, evokes in the receiver a behavioral or physiological reaction that is maladaptive to both the emitter and the receiver. According to this definition, arrestants of *A. loreyi* (pseudohost) represent antimones. However, when neither emitter nor receiver benefit, allelochemicals include following interactions; (1) maladaptive to both emitter and receiver, (2) maladaptive to receiver and nonadaptive to emitter, (3) maladaptive to emitter and nonadaptive to receiver, and (4) nonadaptive to both emitter and receiver. The definition of antimone fits interaction 1, but not interactions 2, 3, and 4. Therefore, we would like to propose a new definition of the term antimone which includes all four interactions. Using the infochemical terminology proposed by Dicke and Sabelis (1988), we define an anti-

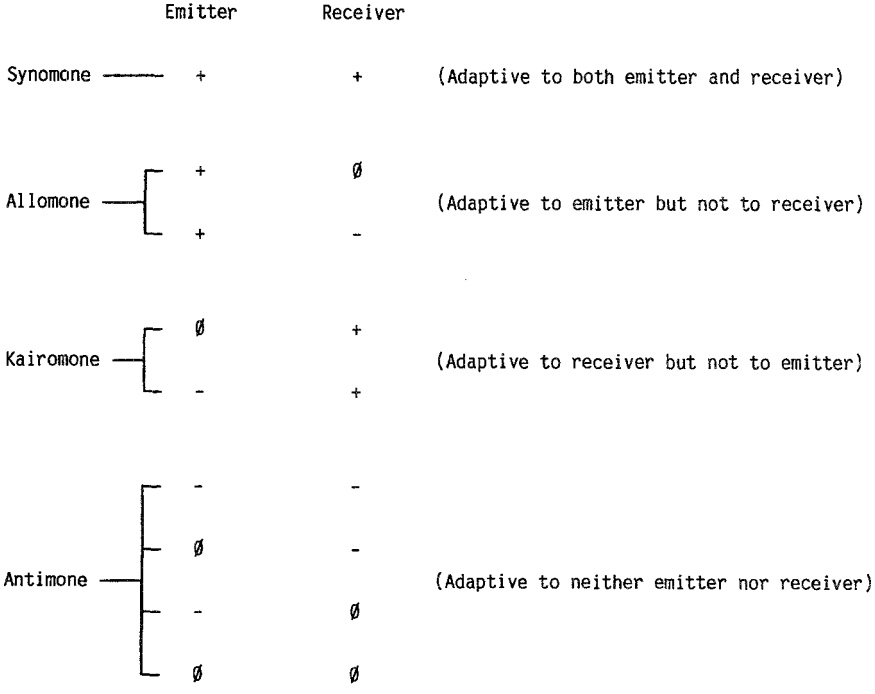


FIG. 1. Structure of allelochemicals; + = adaptive, ø = nonadaptive, − = maladaptive.

mone as an allelochemical that is pertinent to the biology of an organism (organism 1) and that, when it contacts an individual of another species (organism 2), evokes in the receiver a behavioral or physiological response that is adaptively favorable to neither organism 1 nor organism 2. The structure of allelochemicals as proposed here is shown in Figure 1. This definition of antimone provides us the complete structure of allelochemical interactions between organisms.

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REDUCTION OF RODENT FERTILITY BY PLANT CONSUMPTION
With Particular Reference to *Ziziphus spina-christi*

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Abstract—The present study investigates the possibility that plants used in traditional medicine for birth control may also reduce reproduction in their natural herbivores. Ten species of plants utilized by Bedouins for birth control were selected. These were dried, ground, and mixed with the standard diet and offered to female laboratory rats. Six plant species (60%) were found to reduce reproduction rate in white female rats, and the shoots and fruit of one of the effective species, *Ziziphus spina-christi*, when offered to its natural herbivore, *Meriones tristrami*, at the level of 35% of the standard diet, postponed female puberty and significantly reduced offspring survival. Plants that are known to be an effective factor in human birth control may have similar effects on their natural mammalian herbivores. In such cases, when the seeds of the plant are part of the herbivore diet, a certain percentage of the seeds will be dispersed and germinate, while the resulting population control of the animal achieved by its consumption of the seeds will prevent overgrazing, thus maintaining a mutual balance. It is suggested that ethnopharmacological data may assist in uncovering plants that, under the following conditions, have the potential to regulate reproduction in mammalian herbivores (with implications for human reproduction): (1) the plant is an important component of the animal's diet and (2) the active secondary metabolites of the plant directly interact with the physiological systems governing reproduction in the herbivore.

Key Words—Reproduction control, *Ziziphus spina-christi*, *Meriones tristrami*, gerbil, plant–mammal interaction, herbivory.

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INTRODUCTION

A number of studies, under both field and laboratory conditions, suggest that a considerable number of plants have the potential to regulate reproduction in a variety of herbivores (Negus and Berger, 1977; Pinter and Negus, 1965). Many ethnopharmacological and agricultural reports (cf. Farnsworth and Bingel, 1975; Adler and Trainin, 1960; Shapira et al., 1989) also indicate that numerous plants produce secondary metabolites that affect reproduction in humans and various herbivores. Investigation of such plants through direct observations in nature could be extremely laborious and yield only a limited amount of data. It is suggested here that ethnopharmacological evaluation of the effects of plants reputed to inhibit reproduction could shorten the process. Several such studies have been carried out in Israel (Baily and Danin, 1980; Chopra et al., 1960; Crowfoot and Boldensperger, 1932; Fahmy, 1956, 1963; Levy, 1978; Osborne, 1960; Friedman, 1966; Palevitch et al., 1985). In addition, a quantitive ethnopharmacological approach classifying and grading the relative efficiency of medicinal plants of the Negev Desert, as evaluated by their consumers, has recently been published (Friedman et al., 1986). Ten plants reputed to be efficient birth control agents were selected, and primary tests were conducted with them on laboratory white rats. One of the effective plants (*Ziziphus spina-christi*) was then tested on the herbivorous gerbil (*Meriones tristrami*), which feeds on this plant in nature.

METHODS AND MATERIALS

Plants. The stems, leaves, and, in some experiments, fruits and seeds of various medicinal plants were collected from their natural habitats. The material was dried at 45°C for 48 h and then powdered. Weighed amounts of plant powder were mixed with the standard rodent plant material and rodent chow to reach a concentration that was palatable and allowed the animals to maintain their normal weight. The amount of plant material consumed by an animal per day was calculated from the total daily food consumption.

Animals. Young (28 days old) or adult (2–3 months old) Wistar or Charles River rats were maintained under a controlled temperature and lighting regime (14 : 10 light–dark at 22°C) and weighed every two days. Food and water were supplied ad libitum. The animals were offered plant powder mixed with the standard diet for three weeks before mating, followed by 21 days of pregnancy. During the premating period, the stage of the estrus cycle was determined by daily vaginal smears according to the method of Staples and Geils (1965). At the end of this period, when the next proestrus was observed (indicating advent of ovulation), the female rats were paired for mating with proven fertile males.

The day after mating was regarded as the first day of pregnancy if sperm was detected in the vaginal smear. On the 21st day of pregnancy, rats were sacrificed by ether and the number of live, dead, or resorbed fetuses was recorded (the sum of which indicated the number of implantation sites). Since either the pre- or postimplantation phase of pregnancy could account for a decrease in litter size, the number of implantation sites was compared with the number of live fetuses. To determine the number of ovulated ova, the rats were sacrificed on the morning after a successful mating. The two oviducts were flushed out with saline, and the number of ova was counted under a binocular microscope. In order to estimate the ecological relevance of plants that were found to suppress reproduction in white rats, herbivores that were identified as naturally feeding on one of the studied plants were raised in captivity and were offered measured amounts of powder of the tested plant in addition to their normal diet. The response of the wild gerbil *M. tristrami* to a diet including *Z. spina-christi* was examined. Males and females were obtained from the Veterinary Institute, Beit-Dagan. Each pair was maintained separately in a metal cage.

Toxicological Evaluations. To discriminate between direct effects of plant material on the reproductive system and indirect effects resulting from general toxification, toxicological evaluations were performed. Histopathological examination of various organ samples of treated animals (heart, lungs, kidneys, liver, pancreas, stomach, intestines, urinary bulb, adrenal, thymus, thyroid), were carried out according to the method of Bacroft and Stevens (1977). In animals whose reproductive rate was adversely affected, induction of the mixed function oxygenases (MFO) system was measured by determining cytochrome content in liver after homogenizing in buffer phosphate, pH 7.4. The homogenate was centrifuged (12,000*g*) for 30 min and then ultracentrifuged twice (1 h, 105,000*g*). The residue obtained was the microsome fraction. Protein content was determined according to Bradford reaction with bovine serum albumin as a standard (Bradford, 1976). Induction of cytochrome P-450 was measured by difference spectrum (420 nm and 450 nm) between reduced microsomes (with $Na_2S_2O_4$) and reduced microsomes saturated with CO (Omura and Sato, 1964). Cytochrome *b*5 was measured in the same microsome fraction. The difference spectrum (409 nm and 424 nm) is between untreated oxygenated microsomes and reduced ones (Garfinkel, 1958).

RESULTS AND DISCUSSION

Effect of Various Medicinal Plants on Reproduction Rate of Laboratory Rats. Throughout the entire experimental period, the animals maintained their normal weight. The reproductive rates of rats that were offered rodent chow mixed with the various powdered plants is presented in Table 1. The palatability

TABLE 1. EFFECT OF VARIOUS MEDICINAL PLANTS ON REPRODUCTION IN WHITE RATS

Plant tested[a]	Quantity of plant material consumed by white rats		Reproduction (% of control)[b]
	g/kg body wt/day	% of diet	
Pinus halepensis (Pinaceae) (Sd)	60.0 ± 8.5	80	93
Teucrium polium (Labiatae) St + Lv + Fl	21.0 ± 1.5	30	96
Capparis spinosa (Capparaceae) St + Lv	17.5 ± 2.0	15	90
Artemisia herba-alba (Compositae) St + Lv	26.5 ± 3.5	35	90
Peganum harmala (Zygophyllaceae) St + Lv + Sd	3.7 ± 1.5	5	86
Achillea fragrantissima (Compositae) St + Lv + Fl	22.5 ± 4.0	30	84
Retama raetam (Papilionaceae) St + Fr	8.1 ± 1.2	12	81
Whithania somnifera (Solanaceae) St + Lv + Fl + Fr	30.0 ± 5.5	20	80
Ruta chalepensis (Rutaceae) St + Lv	7.5 ± 2.4	10	79
Ziziphus spina-christi (Rhamnaceae) St + Lv + Fr	26.5 ± 4.5	35	75

[a] St = stems, Lv = leaves, Fl = flowers, Fr = fruits, Sd = seeds.
[b] Live fetuses of rats that consumed a plant expressed as % of live fetuses of rats that consumed standard diet (control).

level was determined from the amount of dry plant material consumed daily by the rats (Table 1) as follows: most palatable—seeds of *Pinus halepensis*; moderately palatable—*Ziziphus spina-christi*, *Teucrium polium*, *Artemisia herba-alba*, and *Achillea fragrantissima*; less palatable—*Withania somnifera*, *Capparis spinosa*, *Retama raetam*, *Ruta chalepensis*, and *Paganum harmala*. Of the more palatable plants, only the shoots and fruit of *Z. spina-christi* were

effective in reducing litter size by 25%, when added to the diet at a level of 35%. The less palatable plants were found to be more efficient in decreasing reproduction rate (e.g., *P. harmala*, reduced litter size by 14% when it was offered at 5% of the diet) (Table 1).

Toxicology. Induction of mixed function oxygenases was determined in white rats that were offered one of the following plants: *P. harmale*, *R. roetam*, *W. somnifera*, or *Z. spina-christi*. None of these plants induced additional abnormal histopathological effects, nor was there evidence for induction of cytochromes P-450 and *b*5 in microsomes of liver. It is therefore possible that the examined plants interfered specifically with the reproductive system.

Identification of Stage of Pregnancy Affected by Plant Dry Material. Of five active plants, *Z. spina-christi* induced a preimplantation effect, while the rest (*P. harmala*, *W. somnifera*, *R. raetam*, *R. chalepensis*) interfered with the postimplantation stage (Table 2). Subsequently, we determined the number of fertilized ova in the oviducts of females that consumed *Ziziphus* 12 h after mating. The average number of ova ovulated to the oviducts was reduced by 30% compared to the control (8.0 ± 2.0 compared to 11.8 ± 1.8 ova). This finding supports our assumption that the affected stage was that of preimplantation and, possibly, the ovulation phase.

Selection of Plants that may Regulate Reproduction of Herbivores in Nature. Of the 10 plants examined, six (60%) reduced reproductive success in white rats, thus supporting the significance of traditional medicine as a screening procedure. Although these plants reduced reproduction by various degrees, they did not reveal any significant toxic effect. Although the palatability of most of them was limited, *Z. spina-christi* was highly palatable and at the level of 35% of the diet did not lead to weight loss in the animals, compared to the control. Therefore, herbivores that feed on this species in nature were selected. Several field and laboratory observations supported the assumption that *M. tris-*

TABLE 2. EFFECT OF VARIOUS MEDICINAL PLANTS ON LITTER SIZE OF WHITE RATS

Species	Average number of fetuses per litter ± SEM			Stage affected
	Live	Dead	Total	
Ziziphus spina-christi	7.5 ± 2.7[a]	0.9 ± 1.6	8.4 ± 2.2[a]	Preimplant
Peganum harmala	9.1 ± 1.0[a]	2.4 ± 2.0[a]	11.1 ± 2.0	Postimplant
Withania somnifera	9.2 ± 1.0[a]	1.5 ± 1.0[a]	10.7 ± 1.3	Postimplant
Retama raetam	9.6 ± 1.3[a]	1.5 ± 1.2[a]	11.1 ± 1.8	Postimplant
Ruta chalepensis	8.6 ± 1.3[a]	1.2 ± 0.9[a]	9.8 ± 1.5	Postimplant
Control	10.9 ± 0.3	0.2 ± 0.4	11.1 ± 1.0	

[a] $P \leq 0.05$.

trami feeds in nature on *Z. spina-christi*, based on the following evidence: (1) on the coastal plain, in light soil, burrows of *M. tristrami* were found surrounding trees of *Z. spina-christi*; (2) at the openings of these burrows, considerable concentrations of cracked fruit and pieces of leaves of *Ziziphus* were found; (3) in captivity, *M. tristrami* consumed large amounts of dry leaves and fruit of *Z. spina-christi* and cracked the seeds in a pattern very similar to that found in nature. To evaluate the effect of *Z. spina-christi* on *M. tristrami* in the laboratory, one group of young gerbils (10 animals, 42 days old) was offered *Ziziphus* at a level of 35% of the diet, and a second group, offered 100% standard diet, was used as a control. Animals were weighed and vaginal smears were taken daily. No differences in weight gain by treated and control animals were recorded. The age of vaginal opening in the treated gerbils was delayed by seven days compared with the control animals. In addition, the time lapse between vaginal opening and initiation of first pregnancy was much longer (20 days compared with 13.5 days in the control) (Table 3). A similar response occurred when younger animals (28 days) were offered *Ziziphus* in their diet. In contrast to the response of white rats to *Z. spina-christi*, while *M. tristrami* did not exhibit any remarkable decrease in litter size, only 5% of the litter survived seven days after birth, whereas 60% of the control litter survived. It is inferred therefore that *Z. spina-christi* interfered with reproduction in *M. tristrami* through: (1) delay in vaginal opening, (2) delay in onset of first pregnancy, and (3) a significant decrease in survival rate of young (Table 3).

Identification of the active fraction(s) that interfere with reproduction is

TABLE 3. EFFECT OF *Ziziphus spina-christi* (35% OF DIET) ON VARIOUS REPRODUCTIVE PARAMETERS OF *Meriones tristrami*

Treatment	VO (days)[a]	Weight on VO (g)	Latency (days) between VO and 1st pregnancy	Number of offspring	Survival on the day 7 (%)
Female exposed to male on 42nd day					
Control	48.0 ± 2.0	48.5 ± 5.0	13.0 ± 3.7	5.4 ± 1.1	59.5
Ziziphus	54.0[b] ± 3.3	54.0[b] ± 4.0	20.2 ± 5.5[b]	4.7 ± 0.5	5.0
Female exposed to male on 28th day					
Control	40.5 ± 7.3	40.7 ± 1.9	19.0 ± 5.0	5.4 ± 1.1	53.0
Ziziphus	54.3 ± 3.3	52.3 ± 4.5	38.0 ± 11.0[b]	4.7 ± 0.5	8.0

[a] VO = vaginal opening age.
[b] $P \leq 0.05$.

presently being studied. Our laboratory findings on the effects of *Ziziphus* are based on well-fed animals for which *Ziziphus* constituted 35% of their diet. In nature, the ecological significance of *Ziziphus* in regulating natural reproduction in *Meriones* may be even greater. During the summer months, food sources are scarce and this tree supplies a major part of the diet for this gerbil, which, in turn, disperses some of the *Ziziphus* seeds. The tree is covered with spines that inhibit overgrazing by larger mammals, and in addition its bioactive secondary metabolites control the population size of smaller herbivores, and thus a balance is maintained.

These findings were obtained for only one plant species and cannot be used for more general conclusions. However, the response of *Meriones* to the diet that contained *Z. spina-christi* strengthens our assumption that medicinal plants implicated in birth control may also regulate reproduction of their mammalian herbivores in nature. It is suggested therefore that ethnopharmacological data relating to reproduction should be considered as an important reference for the identification of such plants. Chemical ecology, which usually inspires researchers to evaluate toxic or other effects of medicinal plants, can also benefit from ethnopharmacology. This is particularly pertinent when the traditional utilization of a plant interacts with certain physiological systems, similar to those involved during plant–animal interaction in nature.

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RESPONSES OF LABORATORY-STRAIN MEXICAN FRUIT FLIES, *Anastrepha ludens*,[1] TO COMBINATIONS OF FERMENTING FRUIT ODOR AND MALE-PRODUCED PHEROMONE IN LABORATORY BIOASSAYS

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Abstract—The attractiveness to *A. ludens* adults of a combination of odor of fermented chapote fruit and pheromone was compared to attractiveness of both chapote and pheromone in laboratory bioassays. The chemical treatments were tested on 2-day-old and 10- to 12-day-old (sexually immature and mature, respectively), virgin and mated, nonstarved and starved, males and females during the early afternoon when flies were sexually inactive and the late afternoon when sexually mature flies were sexually active. The chapote–pheromone combination was equal to chapote odor alone in attractiveness to males and mated females. Pheromone partially inhibited attraction of immature females to chapote odor. Chapote odor almost completely inhibited attraction of sexually active females to pheromone. The potential for using a combination of chapote and pheromone as a trap bait is discussed.

Key Words—Attractants, fruit odor, pheromones, Mexican fruit fly, *Anastrapha ludens*, Diptera, Tephritidae, yellow chapote, *Sargentia greggii*, Rutaceae.

INTRODUCTION

Earlier work demonstrated that pheromone produced by male Mexican fruit flies (*Anastrepha ludens* Loew), attracted conspecific, sexually mature, virgin females in laboratory bioassays (Robacker and Hart, 1986; Robacker, 1988)

[1] Diptera: Tephritidae.

and both males and females in the field (Robacker and Wolfenbarger, 1988). Robacker et al. (1990) demonstrated that odor of fermented chapote (*Sargentia greggii* S. Watts) fruit attracted males and females of most ages and at most times of the day. Starved flies responded more strongly than nonstarved ones, indicating that fermented chapote odor functions as a feeding attractant. This finding was not unexpected for two reasons. First, immature chapote fruit is a native larval host of *A. ludens* (Baker et al., 1944). Second, *A. ludens* adults commonly feed on fermenting mangoes, the mature fruit of which is also a larval host (Baker et al., 1944). Robacker et al. (1990) predicted that combinations of fermented chapote odor and pheromone should be more attractive than either alone because each was most attractive to different segments of the adult population.

The objective of the present research was to evaluate attractiveness to *A. ludens* of a combination of fermented chapote odor and pheromone. Attractiveness of chapote, pheromone, and their combination to 2-day-old and 10- to 12-day-old (sexually immature and mature, respectively), virgin and mated, nonstarved and starved, males and females in groups of all males, all females, or both, was tested in laboratory bioassays conducted during both early and late afternoon.

METHODS AND MATERIALS

Insects. Flies were from a culture reared on a meridic diet including torula yeast, carrot, and corncob grit but no fruit. The culture was maintained for at least 70 generations with no wild-fly introductions. Flies were held in 473-ml cylindrical paper cartons with mesh covers from eclosion until testing. Laboratory temperatures ranged from 20 to 25°C and relative humidity ranged from 40 to 70%.

Responses to pheromone and chapote were evaluated for 32 insect-status groups (Table 1). An insect-status group was defined by its sex, age, mating status, potential for interaction with the other sex before and during testing, hunger level, and testing time. Both male and female flies were tested. Ages of flies were 2 and 10–12 days posteclosion. Two-day-old flies were sexually immature and virgin and hereafter are referred to as immature flies. Ten- to 12-day-old flies were sexually mature (Dickens et al., 1982) and either virgin or mated and hereafter are called mature flies. All immature flies were tested in mixed-sex groups containing 80–90 each of males and females. Virgin, mature flies were tested in groups of 80–90 males or females isolated from the other sex since two days posteclosion. Mated, mature flies were tested either in groups of 80–90 males or females isolated from the other sex since eight days posteclosion, or in groups of 60–70 males and 60–70 females held together since

TABLE 1. THIRTY-TWO INSECT-STATUS GROUPS EVALUATED FOR ATTRACTION TO COMBINATION OF ODOR OF FERMENTED CHAPOTE FRUIT AND PHEROMONE RELATIVE TO CHAPOTE AND PHEROMONE ALONE

Sex	Age	Mating status	Other sex present during test?	Test time	Starvation status	Data in:
Male	2 day	virgin	yes (mixed sex)	early PM	fed until test never fed	Figure 1
				late PM	fed until test never fed	
	10–12 day	virgin	no (isolated)	early PM	fed until test 1-day-starved	Figure 2
				late PM	fed until test 1-day-starved	
		mated	no	early PM	fed until test 1-day-starved	
				late PM	fed until test 1-day-starved	
			yes	earlyPM	fed until test 1-day-starved	
				late PM	fed until test 1-day-starved	
Female	2 day	virgin	yes	early PM	fed until test never fed	Figure 1
				late PM	fed until test never fed	
	10–12 day	virgin	no	early PM	fed until test 1-day-starved	Figure 3
				late PM	fed until test 1-day-starved	
		mated	no	early PM	fed until test 1-day-starved	
				late PM	fed until test 1-day-starved	
			yes	early PM	fed until test 1-day-starved	
				late PM	fed until test 1-day-starved	

eclosion. Flies tested in the absence of the other sex were called isolated groups and those tested in the presence of the other sex were called mixed groups. Mating began at five days posteclosion, so that nearly all flies in mixed groups that were going to mate had done so by eight days posteclosion. Previous work indicated that about 90% of the flies mate under these conditions and the other 10% never mate (Robacker et al., 1985). Test times were 1030 hr and 1500 hr

on a photoperiod in which photophase began at 0230 hr and scotophase at 1630 hr. These test times were designated early afternoon and late afternoon in keeping with the shifted photoperiod. Under these conditions, *A. ludens* is sexually active during late afternoon tests but not during early afternoon tests (Robacker and Hart, 1985a). Hunger levels were unstarved and starved. Unstarved flies were fed sugar until the beginning of tests. Starved, mature flies were fed sugar until one day before testing, and starved, immature flies never were fed.

Pheromone and Chapote Extracts. Pheromone was extracted from 7- to 25-day-old (sexually mature) males of *A. ludens* between 1400 and 1600 hr. Males were entrained on the same 0230- to 1630-hr photophase as described above and were sexually active when extracted. Whole males were crushed in hexane (ca. 10 males/ml) and left to soak overnight at room temperature. The hexane was then removed, and the flies were crushed again in a second volume of hexane (ca. 5 males/ml). The two hexane solutions were filtered through a 0.5-μm filter and combined. The resulting extract was concentrated under nitrogen to 1 ME/40 μl of hexane. A ME was defined as the amount of pheromone extracted from one male abdomen by methods used in previous work (Robacker and Hart, 1985b). One ME contained ca. 100 ng of (Z)-3-nonenol, 40 ng of (Z, Z)-3,6-nonadienol, 200 ng of anastrephin (enantiomeric composition unknown), and 700 ng of epianastrephin (enantiomeric composition unknown). Extract was stored at $-20°C$.

Immature chapote fruit was collected near Monterrey, Mexico, during May 1985. The fruit was refrigerated at 5°C in a closed jar until it was extracted during November 1986. The fruit was partially fermented when extracted. Fruit was homogenized in dichloromethane (ca. 0.4 fruits/ml). The homogenate was filtered through a 0.2-μm filter and concentrated under nitrogen to 24 mg-equivalents/40 μl of dichloromethane. A mg-equivalent is defined as the amount of chemicals extracted from 1 mg of fruit. Extract was stored at $-20°C$.

Bioassays. Flies were released into aluminum-framed, aluminum-screened cages (30 cm/side) and taken to a room free of host odor and pheromone at least 2 hr before tests. Light intensity at cage level was ca. 20–25 μE provided by fluorescent lights above the cages.

Cage-top bioassays, modified from previous descriptions (Robacker and Hart, 1984), were used to evaluate responses of the 32 insect-status groups to a combination of fermented chapote odor and pheromone relative to chapote and pheromone separately. The bioassay consisted of placing four filter paper squares (3 × 3 cm) on top of a cage of flies, one filter paper near each corner. One filter paper was loaded with 24 mg-equivalents of chapote extract in 40 μl of dichloromethane, one with 1 ME of pheromone extract in 40 μl of hexane, one with both 24 mg-equivalents of chapote and 1 ME of pheromone in respective solvents, and one with 20 μl of hexane and 20 μl of dichloromethane (blank). The number of flies beneath each paper was counted every 2 min for

10 min. The positions of the four papers were exchanged diagonally across the top of the cage, and the flies were counted for another 10 min. The ratio of the total count beneath the treatment papers to the count beneath the blank was defined as the response index (RI) for each treatment. To prevent excessively high RIs when counts at the blank were low (<4), 4 was used as the blank count when actual counts were 0, 1, 2, or 3.

Evaluations of the 32 insect-status groups by this method were conducted over a five-month period from May to September 1988. The 8–10 replications of the insect-status groups were spread over most of the five-month period to minimize confounding of status groups with time of year. A cage of flies was used for one bioassay, then discarded.

Statistical Analyses. Each insect-status group was a distinct experiment using a randomized complete block design, each block containing one replication of chapote, pheromone, chapote + pheromone, and a solvent blank. Insect-status groups were subjected to factorial analyses of variance (ANOVA) with two factors (chapote and pheromone) tested at two levels each. Data used in ANOVAs were counts at the four papers rather than RIs since the response to the blank was needed in the analyses. Main effects and the interaction of chapote and pheromone were tested using the experimental error mean square. This procedure tested effects of chemical treatments on responses of flies in each insect treatment. Paired *t* tests, in which responses to filter papers with a chemical treatment were compared to blank papers, were conducted in some cases to further evaluate effects of chemical treatments.

Effects of insect-status group on responses of flies to chemical treatments were tested using single degree-of-freedom contrasts (Snedecor and Cochran, 1967) of RI means for a particular chemical from one or more insect-status groups, with RI means for that chemical from one or more other insect-status groups. Each contrast was tested by an F test using a pooled variance from all insect-status groups involved in the contrast. Care was taken not to base conclusions solely on these contrasts since experiments were not designed specifically for these comparisons.

RESULTS AND DISCUSSION

Chapote–Pheromone Combination. The combination of the odor of fermented chapote fruit and pheromone was never more attractive than the chapote odor by itself (Figures 1–3). However, in some cases the combination was less attractive than either chapote odor or pheromone alone as indicated by negative chapote–pheromone interactions.

Among immature flies, one insect-status group had a significant negative chapote–pheromone interaction. That group was starved females tested during

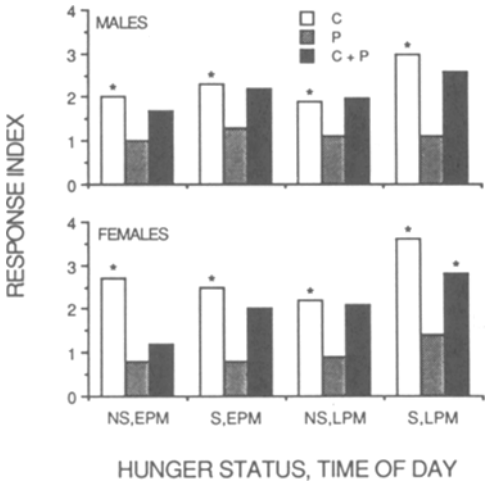


FIG. 1. Attraction of 2-day-old (sexually immature) male and female *A. ludens* to chapote (C), pheromone (P) and their combination (C + P). Flies were tested in mixed-sex groups (M/F = 1). Bars are mean response indices to chemical treatments. Each set of three bars is one insect treatment: NS = nonstarved; S = starved; EPM (early afternoon) and LPM (late afternoon) = test times. *$P < 0.05$ for C and P main effects when placed above C or P bars, or C/P interactions when placed above C + P bars.

the late afternoon ($P < 0.05$) (Figure 1). The response to chapote odor was greater than the response to the combination, indicating that pheromone inhibited response to chapote. This finding was further examined in the other immature female groups. Summed over the four insect-status groups of immature females (Figure 1), mean RIs to the chemical treatments were: chapote = 2.8; pheromone = 1.0; and chapote + pheromone = 2.0. A paired *t* test including all bioassays from the four status groups of females showed that mean response to the chapote + pheromone treatment was significantly lower than to the chapote treatment ($P < 0.001$). The same analysis was conducted on the four status groups of immature males: chapote = 2.3; pheromone = 1.1; and chapote + pheromone = 2.1. The analogous paired *t* test for males was not significant. Thus, pheromone partially inhibited immature females but not males from responding to chapote.

Only one of the 12 status groups of mature males had a significant negative chapote–pheromone interaction. That group was starved virgin males tested during the late afternoon ($P < 0.05$) (Figure 2). As in the above case, the response to chapote odor was greater than the response to the chapote–pheromone combination. Summed over the 12 insect-status groups, mean RIs to the chemical treatments were: chapote = 1.3; pheromone = 0.8; chapote + pher-

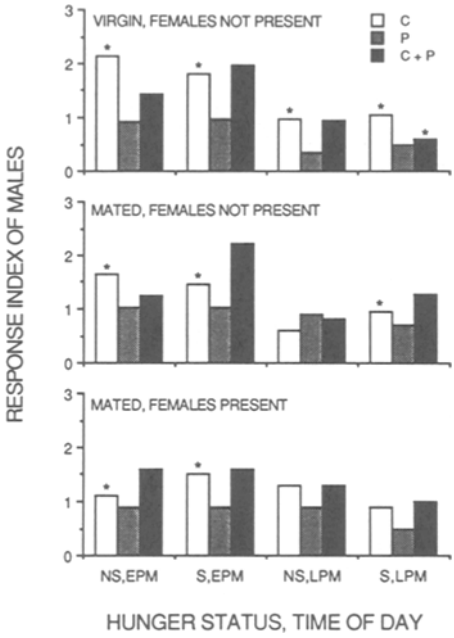


FIG. 2. Attraction of 10- to 12-day-old (sexually mature) male *A. ludens* to chapote (C), pheromone (P) and their combination (C + P). Virgin males were tested in the absence of females; mated males were tested both without and with females present (M/F = 1). Bars are mean response indices to chemical treatments. Each set of three bars is one insect treatment: NS = nonstarved; S = starved; EPM (early afternoon) and LPM (late afternoon) = test times. $*P < 0.05$ for C and P main effects when placed above C or P bars, or C/P interactions when placed above C + P bars.

omone = 1.3. Because the mean difference between the chapote and the chapote + pheromone treatments was 0, the conclusion that pheromone had little or no effect on response of mature males to chapote is most appropriate.

Two of the 12 status groups of mature females had very large negative chapote–pheromone interactions. The two groups were unstarved and starved virgin females tested during the late afternoon ($P < 0.01$ for both groups) (Figure 3). In these two cases, the responses of the females to pheromone were much greater than the responses to the combination. Thus, chapote odor almost completely inhibited response of sexually active, virgin females to pheromone. No other insect-status groups in the mature-females group had significant chapote–pheromone interactions. Summed over the other 10 insect-status groups, mean RIs to the chemical treatments were: chapote = 2.0; pheromone = 1.1; and chapote + pheromone = 2.2.

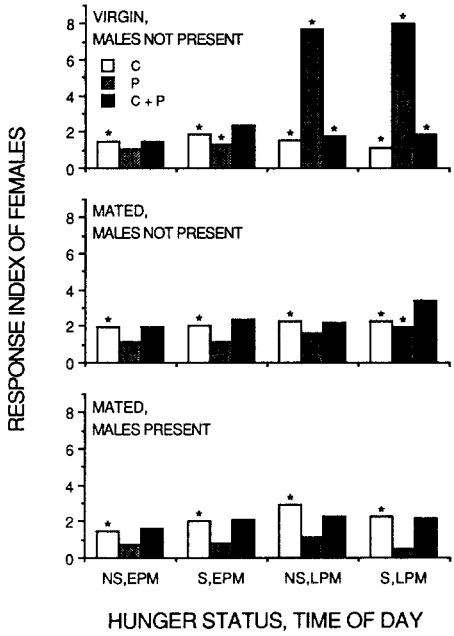


FIG. 3. Attraction of 10- to 12-day-old (sexually mature) female *A. ludens* to chapote (C), pheromone (P) and their combination (C + P). Virgin females were tested in the absence of males; mated females were tested both without and with males present (M/F = 1). Bars are mean response indices to chemical treatments. Each set of three bars is one insect treatment: NS = nonstarved; S = starved; EPM (early afternoon) and LPM (late afternoon) = test times. $*P < 0.05$ for C and P main effects when placed above C or P bars, or C/P interactions when placed above C + P bars.

The above experiments showed that in two cases, immature females and mature, virgin females tested during the late afternoon, chapote odor and pheromone interact with respect to behavior elicited in the test insects. The interaction was neither synergistic nor additive, but inhibitory. In the case of immature females, pheromone inhibited response to chapote since chapote alone was more attractive than the combination of chapote and pheromone. In the case of mature, virgin females tested during the late afternoon, chapote inhibited response to pheromone since pheromone alone was much more attractive than the combination of the two.

Whether these two cases represent different physiological phenomena or two manifestations of the same phenomenon is unknown. A plausible explanation is that each set of active chemical components of the pheromones and of the chapote odors stimulates a different receptor system but the CNS processes information from only one system at a time, the "open" system being deter-

mined by the physiological–motivational state of the animal. Thus, at the time of day when virgin, sexually mature females are sexually active, the CNS processes information from the pheromone receptor system. At this time, information entering a CNS "sexual behavior" processing center from the feeding receptor system may inhibit the output of the sexual behavior center to motor neurons associated with sexual behavior. The result is that sexual attraction to pheromone stops. Conversely, the opposite may be true when the motivational state of the fly is primed for feeding behavior.

Haniotakis and Skyrianos (1981) reported for *Dacus oleae* (Tephritidae) that McPhail traps emitting both female-produced pheromone, attractive to males, and the feeding attractant Entomozyl, attractive to both sexes, captured fewer males and fewer females than McPhail traps emitting only feeding attractant. Haniotakis and Vassiliou-Waite (1987), using McPhail traps baited with pheromone and the feeding attractant ammonia, obtained the same reduction in male *D. oleae* captures as previously, but obtained an increase in female captures compared to captures by traps emitting only ammonia or only pheromone. These results are similar to those in our work in that the combination of a feeding attractant and a sexual attractant was less attractive than the feeding attractant alone, at least to the sex (males) most responsive to the pheromone. Thus, it appeared in their work that pheromone inhibited response to the feeding attractant. Their results differ from ours with regard to the sex affected by the negative feeding attractant–pheromone interaction and by the apparent lack of an inhibitory effect by the feeding attractant on response to pheromone. However, the latter effect may have occurred undetected since their field experiments were not designed to distinguish sexually inactive males from the sexually active males most likely to be affected by such an interaction.

In various non-Hawaiian *Drosophila* species, combinations of aggregation pheromones and feeding attractants such as fermenting host bark, rearing medium, or acetone usually attract two- to sixfold more flies than either pheromone or feeding attractant alone (Bartelt et al., 1985a,b, 1986, 1988; Schaner et al., 1987). These synergistic effects contrast sharply with the inhibitory effects in the two tephritid species described above. Differences in behavior between the Tephritidae and the Drosophilidae probably derive from differences in mating systems. *A. ludens* mates most often on the undersides of leaves away from food (Robacker and Hart, 1985a). This non-resource-based mating system suggests that the flies separate feeding and mating in time and space and would not be attracted in nature to both feeding and mating stimuli at the same time. Most non-Hawaiian *Drosophila*, on the other hand, mate while aggregated at feeding sites (Spieth, 1974; Burk, 1981). Combinations of food odor and pheromone would be a natural consequence of such a food-resource-based mating system, and it is not surprising that food odor and pheromone act together to attract these *Drosophila*. Unfortunately, the mating system of *D. oleae*, which involves

both male- and female-produced pheromones, is little understood, making comparisons with *A. ludens* or the *Drosophila* difficult (Haniotakis, 1974; Haniotakis et al., 1986; Fletcher, 1987).

Responses to Chapote. Robacker et al. (1990) reported effects of age, test time, and starvation on attractiveness of fermented chapote fruit odor to *A. ludens*. Although treatments were not identical, results of that work and the present study largely agree. Robacker et al. (1990), however, did not evaluate effects of mating status. The present work showed that mating status (virgin vs. mated) did not affect responses by either sex to fermented-chapote odor (Figures 2 and 3).

Responses to Pheromone Extract. Results generally agree with our earlier pheromone studies (Robacker and Hart, 1985b; Robacker et al., 1985, 1990). In addition, the present study demonstrates that one day of starvation does not affect response to pheromone by sexually active females and that sexually mature, virgin females begin to respond to pheromone as early as 6 hr before scotophase (early afternoon test time) (Figure 3).

The present study also showed that pheromone was slightly attractive to the starved, mature, mated female group tested during the late afternoon (Figure 3). This may have been due to the presence of some females that did not mate during their eight-day access period to males or to recurrence of sexual drive in mated females (Robacker et al., 1985). Pheromone was not attractive to flies in any other individual insect-status groups.

Attraction vs. Arrest Responses to Extracts. Responses of flies to both chapote extract and pheromone extract were attraction responses. As evidence, flies did not feed from the papers in either case. Moreover, the attractiveness of the papers decreased during the bioassays as the volatile chemicals evaporated.

Effects of Sexual Activity on Response of Males to Filter Papers. Figure 2 shows that sexually mature males tested during the late afternoon (sexually active males) responded less than sexually mature males tested during the early afternoon (sexually inactive males) to chapote ($P < 0.01$), a finding reported previously (Robacker et al., 1990). In addition, sexually active males spent less time under blank filter papers than sexually inactive males. Summed over mating status and starvation status groups, counts of mature males beneath blank papers were: early afternoon = 4.1 flies per test; late afternoon = 2.0 flies per test. The late afternoon counts were significantly lower than the early afternoon counts ($P < 0.01$). Time of day did not affect responses of other insect treatments to the blank filter papers.

The reason sexually active males apparently avoided the undersides of filter papers is unknown. However, it is noteworthy that sexually active males preferred undersides of citrus leaves in previous work, especially for calling (Robacker and Hart, 1985a).

Chapote–Pheromone Combinations as Trap Baits. The data do not dem-

onstrate that chapote–pheromone combinations are more attractive than either alone, but it would be premature to conclude that combinations would yield similar results in the field. For example, pheromone was not very attractive to males in the present laboratory study, but Robacker and Wolfenbarger (1988) showed that pheromone attracted males and females equally in the field. Possibly, chapote–pheromone combinations may have additive effects on captures of males in the field because of attractiveness of both chapote and pheromone and absence of a negative chapote–pheromone interaction. While a similar scenario seems less likely for females, the increase in male captures may offset any decrease in female captures caused by inhibitory effects of chapote and pheromone on each other.

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EVIDENCE FOR ALLELOPATHY BY TREE-OF-HEAVEN *(Ailanthus altissima)*

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Abstract—*Ailanthus altissima* (Mill.) Swingle contains one or more phytotoxic compounds in roots and leaves. Activity is higher in roots, where it occurs primarily in the bark. Powdered root bark and leaflets strongly inhibited growth of garden cress (*Lepidium sativum* L.) when mixed with soil in Petri dishes (ID_{50} values = 0.03 g root bark, 0.6 g leaflet/dish). The toxic material was readily extracted by methanol but not dichloromethane. Pieces of root bark mixed with soil at 2, 1, and 0.5 g/pot reduced cress biomass in the greenhouse, whereas methanol-extracted root bark did not. The inhibitory effect of *Ailanthus* tissues in soil was short-lived ($\leq$4 weeks in pots in greenhouse, $\leq$3 days in Petri dishes in laboratory). Inhibition by root bark was sometimes superseded by stimulation. Fresh *Ailanthus* root segments placed in or on soil reduced growth of nearby cress seedlings. Fine roots were more inhibitory than coarse, and inhibition became more pronounced with increased time of soil exposure to roots. Soil collected near *Ailanthus* roots in the field supported reduced radicle growth of cress compared to control soil. In contrast, stemflow from *Ailanthus* trees stimulated cress growth. The results suggest allelopathy caused by toxin exudation from roots may contribute to the aggressiveness and persistence of *Ailanthus* in certain habitats.

Key Words—*Ailanthus altissima*, tree-of-heaven, Simarubaceae, allelopathy, allelochemicals, phytotoxicity, root exudates, succession.

INTRODUCTION

Ailanthus altissima (Simarubaceae) is a rapidly growing, extremely aggressive tree. Introduced in 1784 from Asia, it is now widely naturalized and occurs throughout much of the United States (Munz and Keck, 1959; Fernald, 1970).

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Ailanthus is remarkable in its ability to rapidly invade and colonize disturbed, often seemingly harsh, habitats. In southern Pennsylvania, New York, and Connecticut, *Ailanthus* sometimes invades recently abandoned fields, suppressing growth of other tree species and forming nearly pure stands (Mergen, 1959; Newton, 1986). *Ailanthus* is also very tolerant of urban conditions and is one of the most abundant "volunteer" trees in northeastern U.S. cities such as New York and Philadelphia (Little, 1974; Newton 1986). It readily invades and thrives along roadsides, in cracks of sidewalks and pavement, in vacant lots, and almost anywhere else sufficient dust or soil has accumulated.

A number of characteristics undoubtedly contribute to the fitness of *Ailanthus*. It grows very rapidly and readily overtops most competitors. *Ailanthus* reproduces both sexually, with abundant samaras, and vegetatively, with sprouts from wide-ranging lateral roots (Pan and Bassuk, 1986). Root sprouts may remain suppressed for a considerable time until conditions become favorable. Lateral root growth is tolerant of, or even enhanced by, soil compaction (Pan and Bassuk, 1985). These attributes alone, however, appear inadequate to explain the invasiveness and tenacity of *Ailanthus* at certain sites.

An arsenal of biochemical defenses may also contribute to the fitness of *Ailanthus*. Many plants produce secondary compounds capable of inhibiting other plants, pathogens, or herbivores (Whittaker and Feeny, 1971; Harborne, 1988; Niemeyer, 1988). Yang and Tang (1988), in a compilation of plants used for pest control in China, report *Ailanthus* leaves and root bark have been used on the insects *Pieris rapae*, *Platyedra gossypiella*, and aphids. Aqueous extracts of *Ailanthus* foliage inhibit germination and growth of other plant species (Mergen, 1959; Heisey and Delwiche, 1983). Heisey (1990) demonstrated one or more phytotoxic allelochemicals were present in *Ailanthus* foliage, bark of roots and trunk, and certain other tissues. Root bark was most phytotoxic.

Much evidence suggests allelopathy is important in the establishment and persistence of certain plant species (Silverstein and Simeone, 1983; Rice, 1984; Putnam and Tang, 1986). *Ailanthus* is an early successional tree intolerant of heavy shading. It sometimes forms nearly pure stands that appear resistant to invasion by later successional species (Mergen, 1959). Because of the strong phytotoxic activity of certain *Ailanthus* tissues, I hypothesized allelopathy may contribute to the aggressive interference exhibited by this species. As stated by Harper (1977) and emphasized by others (e.g., Stowe, 1979; Fuerst and Putnam, 1983; Heisey and Delwiche, 1985), however, the mere presence of phytotoxins in plants does not prove the occurrence of allelopathy under natural conditions. The purpose of this paper is to quantify, especially in soil, the phytotoxicity of allelochemicals produced by *Ailanthus* and to evaluate their potential for allelopathy under natural conditions.

METHODS AND MATERIALS

General Methods. The phytotoxicity of *Ailanthus* tissues, soils, and stemflow was quantified with bioassays on seeds of garden cress. Most bioassays were done in 10 × 1.5-cm plastic Petri dishes with 10–15 seeds per dish. Garden cress is a sensitive indicator of phytotoxic compounds and has been widely used in studies of allelopathic and herbicidal natural products (Lehle and Putnam, 1982; Weston et al., 1987; Heisey and Putnam, 1990). Bioassays in Petri dishes were incubated at 28°C in darkness. Cress radicle growth was determined after three days and used as an indicator of biological activity. Radicle elongation is a more sensitive assay of allelochemicals than germination (Barnes and Putnam, 1987; Leather and Einhellig, 1988).

Ailanthus tissues were collected from a 20-year-old *Ailanthus* grove on the Calder Conservation and Ecology Center in southeastern New York. Soil in the grove, as well as that collected nearby for use in bioassays, was of the Chatfield series (typic Dystrochrepts, coarse-loamy, mixed, mesic). Soil was sieved through a 2.38-mm mesh screen before use. *Ailanthus* root bark was obtained from a 19-year-old tree (20 cm dbh) on May 29, 1987. Roots were excavated and rinsed with tap water with light brushing to remove soil. Bark was peeled from the roots and stored at −27°C until needed. Leaflets were collected on several dates, air-dried, and similarly stored. Air-drying was at room temperature (ca. 23°C) and oven-drying at 55 ± 5°C. Samples were ground in a Wiley mill with a 20-mesh screen. Tissues were extracted with dichloromethane or methanol in a Soxhlet apparatus for 48 hr or more, with solvent cycling every 20–35 min.

Toxicity of Ailanthus *Tissues in Soil in Petri Dishes.* An initial investigation was conducted to quantify the toxicity of *Ailanthus* leaflets and root bark in soil and to determine whether the phytotoxins could be extracted from the tissues with dichloromethane or methanol. Air-dried *Ailanthus* root bark and leaflets (collected November 9, 1987, after having fallen from trees) were extracted with dichloromethane. The tissues were oven-dried 48 hr or more to remove all dichloromethane and milled before or after extraction. Dichloromethane was ineffective in extracting the toxins. Subsequently, dichloromethane-extracted root bark and leaflets (taken from trees on September 11, 1987) were reextracted with methanol. The extracted tissues were oven-dried at 80–85°C to drive off all methanol and milled before or after extraction.

Bioassays were done with 60 g of air-dry soil in Petri dishes. Ground *Ailanthus* tissues (0–1 g root bark, 0–2 g leaflets) were mixed into the upper third of soil and covered with 25 g more soil to fill the dishes. The bioassays were thoroughly moistened with 30 ml deionized water, a sheet of Whatman No. 1

or 2 filter paper was placed over the soil, and cress seeds were distributed thereon. The dishes were covered, inverted, and incubated.

Toxicity of Ailanthus *Tissues in Soil in Pots.* This investigation further evaluated the allelopathic activity of *Ailanthus* root bark and leaflets in soil, under conditions more comparable to those in the field. Plastic pots (9 × 6 cm diam.) were partially filled with 170 g of air-dry soil. This soil was covered 1.5 cm deep with 90 g more soil containing 0.5, 1, or 2 g oven-dry root bark (50–100 mm^2 pieces) or leaflets (25–50 mm^2 flakes). A second group of pots received *Ailanthus* tissues from which the toxins had been extracted with methanol or dichloromethane followed by methanol. Control pots received no *Ailanthus* tissues. Cress seeds, 20 per pot, were planted 0.5 cm deep and irrigated from above with tap water. The experimental design was a randomized complete block with 10 replications (except treatments receiving extracted leaflets were replicated only five times). Seedling emergence was recorded after five or six days. The shoots were harvested after 21 days, oven-dried, and weighed.

The phytotoxicity of *Ailanthus* root bark in soil appeared to decrease over time during this investigation. To obtain more information regarding persistence of toxicity, the pots after harvest were allowed to dry in the greenhouse for one week. Those pots previously amended with either 2 g or 0 g of the *Ailanthus* tissues were then replanted and assayed again as before.

Degradation of Toxicity over Time in Soil. The previous experiment suggested toxins from *Ailanthus* root bark are active for only a short time in nonsterile soil. An investigation was designed specifically to examine the persistence of toxicity. Petri dishes were filled with 74 g of air-dry soil. Autoclaved (25 min, 15 psi), ground *Ailanthus* root bark (0.2 g) or leaflet (1.5 g) was incorporated into the upper third of the soil. These amounts of *Ailanthus* tissues were chosen on the basis of results shown in Figures 1 and 2 to inhibit cress radicle growth to $\leq 10\%$ of controls. The controls lacked *Ailanthus* powder but were otherwise identical to treatments. The dishes were moistened with 20 ml of deionized water and incubated at 28°C for time intervals ranging from 0 to 29 days. The dishes were prepared such that all incubations ended simultaneously. After incubation, a sheet of sterile filter paper was placed over the soil, cress seeds were added, and the dishes were inverted and incubated three days more. The experimental design was a randomized complete block with three or four replications.

Release of Toxicity from Ailanthus *Roots.* This investigation was designed to determine whether phytotoxins are exuded from *Ailanthus* roots into soil. Living *Ailanthus* roots were collected in the field on July 13, 1987. Roots of two diameter classes were selected and cut to desired length (fine = diam. ≤ 3 mm, length 2–3 cm; coarse = diam. 8 mm, length 8 cm). Petri dishes were filled with 60 g field moist soil. Fine roots (2 g fresh wt) were distributed on

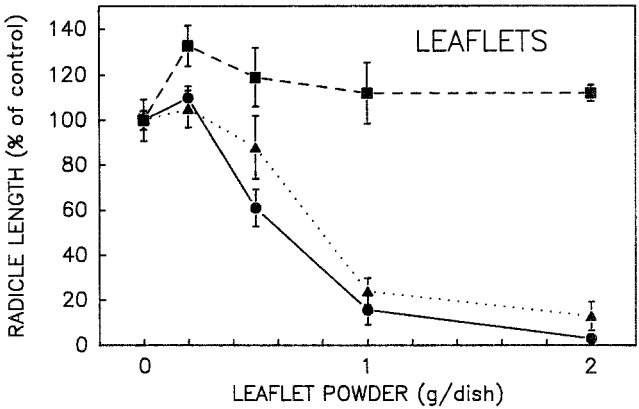


FIG. 1. Radicle growth of garden cress in Petri dishes containing soil and nonextracted (solid line), dichloromethane-extracted (dotted line), or methanol–dichloromethane-extracted (dashed line) *Ailanthus* leaflet powder. Data are mean ± SE expressed as percentage of control; where bars are absent, SE is ≤ height of symbol.

the soil surface, or one coarse root segment (6–9 g fresh wt) was pressed into the soil until level with the surface. Controls received soil but no roots. The dishes were placed in a refrigerator (0 ± 4°C) for times ranging from 0 to 13 days to allow exudation and diffusion of toxins from the roots while simulta-

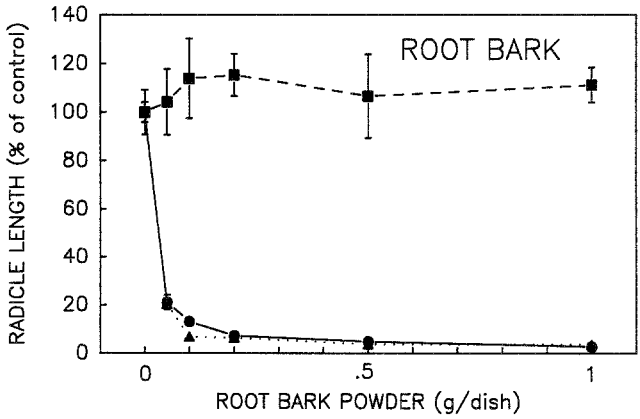


FIG. 2. Radicle growth of garden cress in Petri dishes containing soil and nonextracted (solid line), dichloromethane-extracted (dotted line), or methanol–dichloromethane-extracted (dashed line) *Ailanthus* root bark powder. Data are mean ± SE expressed as percentage of control; where bars are absent, SE is ≤ height of symbol.

neously retarding microbial activity. The dishes were then taken from refrigeration, and cress seeds were placed in two rows 1–2 cm apart across the soil. In dishes with coarse root, the seeds were placed 2–5 mm from both sides of the root. The bioassays were incubated at 28°C as a completely randomized design with four replicates.

Toxicity of Field Soil near Ailanthus *Roots.* The previous investigation indicated *Ailanthus* roots release toxicity to soil under laboratory conditions. An investigation was undertaken to examine whether soil near *Ailanthus* roots in the field becomes inhibitory. Soil samples of approximately equal volume were collected adjacent to and within 2 cm of living roots (typically ≥ 1 cm diam.) of 10 *Ailanthus* trees (dbh $\geq$ 10 cm). The samples were combined and mixed. Control soil was collected from 10 spots in a wooded area 100–200 m away containing few *Ailanthus* trees. Moisture content was determined by drying at 105°C; pH was measured on a 1-g moist soil–2.5-ml deionized water slurry. Sixty grams of the field moist soils were weighed into Petri dishes, irrigated with 10 ml deionized water, and covered with a sheet of Whatman No. 1 paper. Cress seeds were placed on the filter paper. The experimental design was a randomized complete block with 10 replicates. The bioassay was performed twice, the second time seven days after the first. The soils were refrigerated (5°C) between assays.

Biological Activity of Ailanthus *Stemflow.* The purpose of this investigation was to determine whether rainwater becomes toxic as it travels down the foliage, branches, and trunk of *Ailanthus* trees. Polyurethane stemflow collectors (Likens and Eaton, 1970) were placed around five *Ailanthus* trees (9, 13, 17, 19, 28 cm dbh). Three funnel bottles were placed in open areas nearby to collect control precipitation. Deionized water acidified with sulfuric acid to pH 3.8, the pH of stemflow and rainfall collected, was used as an additional control. Precipitation was collected after a 1.5-cm rain on June 2–3, 1987. Petri dishes (6 × 1.5 cm) containing one sheet Whatman No. 1 paper were seeded with cress and moistened with 2 ml of precipitation or deionized water. The experimental design was a randomized complete block with four replicates.

RESULTS AND DISCUSSION

Toxicity of Ailanthus *Tissues in Soil in Petri Dishes. Ailanthus* root bark and leaflets mixed with soil both inhibited radicle elongation of garden cress seedlings (Figures 1 and 2). Root bark was about 20 times more toxic than leaflets, with ID_{50} values (dose inhibiting radicle elongation to 50% of controls) of approximately 0.6 g tissue/dish for leaflets and 0.03 g tissue/dish for root bark. As little as 0.1–0.2 g root bark/dish reduced cress radicle growth to less

than 10% of the control, whereas more than 1 g leaflet/dish was required for a similar level of inhibition.

The toxicity in *Ailanthus* leaflets and root bark was readily extractable with methanol but not with dichloromethane. Dichloromethane extraction of root bark and leaflets did not appreciably reduce phytotoxicity compared to nonextracted tissues (Figures 1 and 2). Methanol extraction, in contrast, greatly reduced toxicity. In all treatments with methanol-extracted root bark or leaflets, cress radicle elongation was similar to, or greater than, controls receiving no *Ailanthus* tissue.

Considerable attention currently is directed toward using allelopathic plants and allelochemicals for weed control in agroecosystems (Einhellig and Leather, 1988; Putnam, 1988). It is interesting to speculate on the use of powdered *Ailanthus* root bark as a herbicidal soil treatment. The surface area of the Petri dishes used in this bioassay was 61 cm^2 (6.1×10^{-7} hectares). If we consider 0.1 g root bark powder per dish as providing sufficient herbicidal activity, then 164 kg/ha would be needed. This is a massive dose compared to current applications of synthetic herbicides (typically 4 kg/ha or less). However, since the herbicidal compounds in *Ailanthus* root bark probably represent only a small proportion of the total weight, the required application of purified toxin would be much lower. For example, if the herbicidal compounds constitute 1%, a reasonable expectation, then only 1.6 kg/ha of active ingredient would be required. These calculations suggest phytotoxins in *Ailanthus* root bark are sufficiently active to justify further investigations into their potential use as herbicides.

Toxicity of Ailanthus *Tissues in Soil in Pots. Ailanthus* root bark significantly reduced growth of cress in the greenhouse (Table 1). The toxins exerted their effect both by reducing emergence of seedlings and by decreasing vigor of emerged seedlings. Two grams per pot drastically inhibited seedling emergence and reduced cress shoot biomass to 5% of the control receiving no *Ailanthus* tissue. Even the lowest dose, 0.5 g/pot, strongly inhibited seedling emergence and biomass production. The percentage of seedlings surviving to harvest 21 days after planting did not differ appreciably from the percentage already emerged by days 5 or 6.

Methanol-extracted root bark did not inhibit cress growth, and even stimulated it in some cases, compared to the control receiving no *Ailanthus* tissue. It also had little effect on seedling emergence or survival. Two reasons may explain the stimulation. Stimulatory organic compounds, or perhaps small amounts of the inhibitory compounds, may remain in the *Ailanthus* root bark despite solvent extraction. Certain herbicidal compounds stimulate plant growth at very low, subtoxic concentrations (Ries, 1976). Hence, solvent extraction may have reduced the concentration of the inhibitors in *Ailanthus* root bark to

TABLE 1. GROWTH OF GARDEN CRESS IN POTS CONTAINING NONEXTRACTED *Ailanthus* ROOT BARK (NER), METHANOL-EXTRACTED ROOT BARK (ER), OR NO ROOT BARK (CONT)

	Treatment (g/pot)						
	NER			ER			CONT
	0.5	1	2	0.5	1	2	0
Biomass							
mg per pot[a]	92c	42d	8e	205a	191ab	207a	170b
% of control	55c	25d	5e	123a	114ab	124a	100b
Emergence[b] (%)	39	21	5	86	88	91	92
Survival[c] (%)	36	20	6	82	83	84	90

[a] Means followed by different letters differ at $P \leq 0.05$ in Duncan's multiple-range test.
[b] Emergence = (seedlings emerged 5–6 days after planting/20 seeds per pot) × 100.
[c] Survival = (seedlings alive 21 days after planting/20 seeds per pot) × 100.

a stimulatory level. Another possibility is that inorganic nutrients in extracted *Ailanthus* root bark may have stimulated cress growth after the allelochemicals were eliminated by solvent extraction. Additive or synergistic effects are also possible.

Harper (1977) criticizes some research on allelopathy for using inappropriate controls. In such investigations, leaves or other tissues of plants suspected of being allelopathic are mixed with soil and bioassayed. For controls, peat, filter paper, or other microbially refractory materials are sometimes used. The addition of labile organic matter, such as leaves, to soil typically stimulates soil microbial populations, causing rapid immobilization of available nitrogen and possibly other plant nutrients. Plants in such treatments would thus suffer nutrient deficiency and reduced growth. More slowly decomposed organic matter, such as peat, does not have the same effect. Hence, unless appropriate controls are used, separating allelopathy from nutrient deficiency is difficult.

The ideal control would be identical in every way, except for lacking allelochemicals, to the treatments. This was my rationale for using methanol-extracted tissues. Methanol extraction removed most of the toxicity without appreciably altering the tissues otherwise. Nutrient immobilization did not appear to be a serious complication, because cress biomass in treatments receiving methanol-extracted tissues was comparable to, or higher than, in the control. Since methanol-extracted root bark stimulated cress growth relative to the control, the inhibitory effect of nonextracted root bark appears even greater when compared to the treatments receiving extracted root bark. Therefore, the

evidence is strong that *Ailanthus* root bark contains one or more very potent phytotoxins.

The inhibitory effect of *Ailanthus* root bark in soil is ephemeral. When cress was replanted in soil that had contained 2 g of nonextracted *Ailanthus* root bark for 28 days and was previously strongly inhibitory, inhibition was now replaced by stimulation (Table 2). Methanol-extracted root bark, which stimulated the first cress planting (Table 1), also stimulated the second. These results indicate the inhibitors are rapidly altered in soil, probably by microorganisms. The increased biomass production, relative to the control receiving no tissue, again suggests stimulatory materials are present in *Ailanthus* root bark or that inhibitor concentrations have fallen to stimulatory levels.

Ailanthus leaflets in soil were much less inhibitory than root bark (Table 3), but their inhibitory effect lasted longer (Table 4). In the first planting, the only treatment causing a statistically significant reduction in cress biomass was 2 g nonextracted leaflets/pot, which reduced biomass to 64% of the control. Extracted leaflets applied at 2 g/pot reduced cress biomass to 75%, but the difference was not significant. A similar pattern occurred in the second planting, where 2 g/pot significantly reduced cress biomass to 63% for nonextracted leaflets and 80% for extracted leaflets. Seedling emergence and survival did not differ appreciably between treatments and their corresponding control.

Degradation of Toxicity over Time in Soil. The phytotoxicity of *Ailanthus* root bark and leaflets decreased rapidly over time in soil (Figure 3). Root bark and leaflets were both very inhibitory immediately after incorporation into nonsterile soil, reducing radicle growth of cress to near zero. Leaflets (1.5 g/pot) remained highly toxic for three days but were not inhibitory by seven days.

TABLE 2. GROWTH OF GARDEN CRESS REPLANTED IN POTS PREVIOUSLY USED FOR TABLE 1 CONTAINING NONEXTRACTED *Ailanthus* ROOT BARK (NER), METHANOL–DICHLOROMETHANE-EXTRACTED ROOT BARK (ER), OR NO ROOT BARK (CONT)[a]

	Treatment (g/pot)		
	NER, 2	ER, 2	CONT, 0
Biomass			
mg per pot[b]	367a	199b	102c
% of control)	360a	195b	100c
Emergence (%)	85	73	77
Survival (%)	92	75	81

[a] Cress was replanted 28 days after *Ailanthus* root bark had been added to soil. Treatments and parameters as in Table 1.

[b] Means followed by different letters differ at $P \leq 0.05$ in Duncan's multiple-range test.

TABLE 3. GROWTH OF GARDEN CRESS IN POTS CONTAINING NONEXTRACTED *Ailanthus* LEAFLETS (NEL), METHANOL–DICHLOROMETHANE-EXTRACTED LEAFLETS (EL), OR NO LEAFLETS (CONT)

	Treatment (g/pot)						
	NEL			EL			CONT
	0.5	1	2	0.5	1	2	0
Biomass							
mg per pot[a]	136ab	136ab	107b	132ab	142ab	126ab	167a
% of control	81ab	81ab	64b	79ab	85ab	75ab	100a
Emergence[b] (%)	94	88	93	90	94	96	92
Survival[b] (%)	92	82	90	86	88	92	90

[a] Means followed by different letters differ at $P \leq 0.05$ in Duncan's multiple-range test, as modified by Kramer (1956) for unequal replication.
[b] Defined in Table 1.

After two weeks in soil, leaflets stimulated cress radicle growth. Root bark (0.2 g/pot) lost its toxicity even more rapidly than leaflets. After the first day, it had little effect or was slightly stimulatory. The pattern of strong initial inhibition followed by stimulation is similar to that observed in the greenhouse experiment with *Ailanthus* root bark. Leaflets, in contrast, were stimulatory after two weeks in the present experiment but remained inhibitory for at least four weeks in the greenhouse. These results further support the idea that phytotoxins from *Ai-*

TABLE 4. GROWTH OF GARDEN CRESS REPLANTED IN POTS PREVIOUSLY USED FOR TABLE 3 CONTAINING NONEXTRACTED *Ailanthus* LEAFLETS (NEL), METHANOL–DICHLOROMETHANE-EXTRACTED LEAFLETS (EL), OR NO LEAFLETS (CONT)[a]

	Treatment (g/pot)		
	NEL, 2	EL, 2	CONT, 0
Biomass			
mg per pot[b]	64c	81b	102a
% of control	63c	80b	100a
Emergence (%)	82	76	77
Survival (%)	84	87	81

[a] Cress was replanted 28 days after *Ailanthus* leaflets had been added to soil. Treatments and parameters as in Table 1.
[b] Means followed by different letters differ at $P \leq 0.05$ in Duncan's multiple-range test, as modified by Kramer (1956).

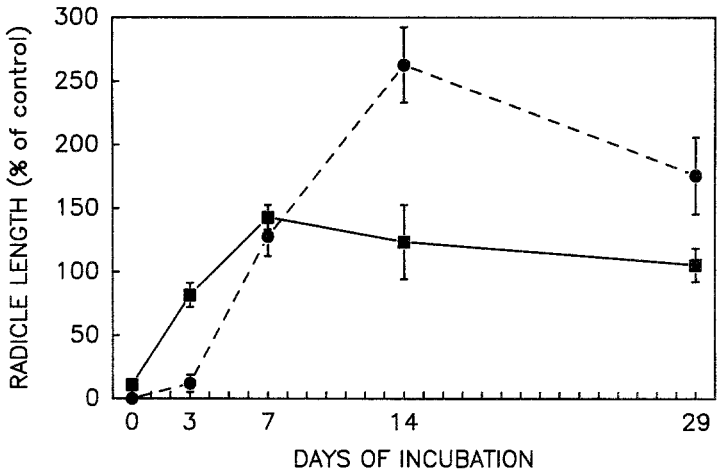


FIG. 3. Radicle growth of garden cress in Petri dishes containing soil amended with 0.2 g *Ailanthus* root bark powder (solid line) or 1.5 g *Ailanthus* leaflet powder (dashed line) and incubated for 0–29 days at 28°C before planting cress seeds. Data are mean ± SE expressed as percentage of simultaneous controls; where bars are absent, SE is ≤ height of symbol.

lanthus roots and leaflets do not persist long in warm soil and suggest soil microorganisms are involved in their degradation. Schmidt (1988) came to a similar conclusion regarding the persistence of juglone, an allelochemical produced by black walnut (*Juglans nigra* L.).

Autoclaving did not appreciably reduce the toxicity of *Ailanthus* leaflets or root bark, indicating the inhibitors are relatively heat-stable. Autoclaved tissues (1.5 g leaflet/dish or 0.2 g root bark/dish) reduced cress radicle growth to 0% and 11%, respectively, of controls (Figure 3). This is nearly identical to the effect of nonautoclaved tissues (Figures 1 and 2).

Obviously, the persistence of toxins in soil depends on the amount initially present and on soil biological processes. If microbial activity is important in degrading the toxins, then larger applications would lead to longer persistence, and smaller doses would disappear more rapidly. In the case of cinnamic acid, an allelochemical produced by certain plants, a concentration of 0.1 mg/g soil strongly inhibited plant growth for less than two days, whereas 1 mg/g soil was inhibitory for 7–14 days (Bonner, 1946). Thus, before the allelopathic potential of toxins from *Ailanthus* can be adequately assessed, the quantity of toxin released from roots, and the effects of soil microorganisms thereon, must be known.

Toxin Release from Ailanthus *Roots. Ailanthus* roots in contact with soil reduced growth of cress nearby (Figure 4). Fine roots appeared to release toxins

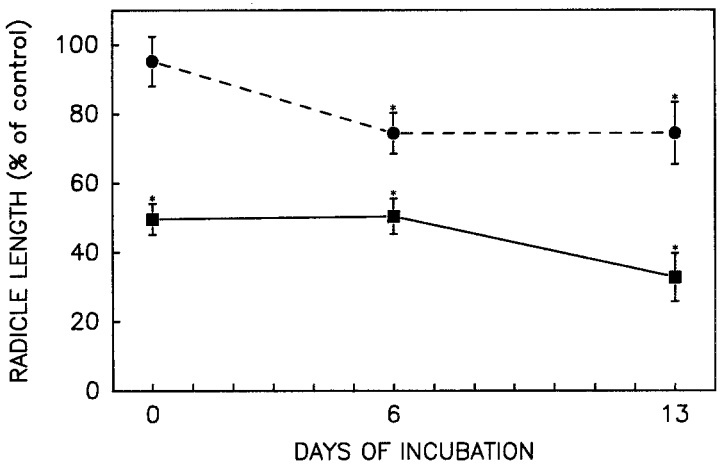


FIG. 4. Radicle growth of garden cress in Petri dishes containing soil and fine (≤ 3 mm diam.) *Ailanthus* roots on soil surface (solid line) or one segment of coarse *Ailanthus* root (ca. 8 mm diam.) pressed into soil (dashed line). Data are mean $\pm$ SE expressed as percentage of simultaneous controls. Data points accompanied by asterisk differ from corresponding control ($P \leq 0.05$) in t test.

more rapidly and in greater amount. When tested immediately after addition of roots, soil containing fine roots (≤ 3 mm diam.) depressed cress growth to half that of the control, whereas coarse roots (ca. 8 mm diam.) had no effect. Both fine and coarse roots inhibited cress growth after soil had been exposed six days, but fine roots remained more inhibitory. This pattern persisted to the end of the incubation period (13 days), with inhibition being greatest then.

This experiment provides strong evidence that toxins are released from *Ailanthus* roots into soil. The previous experiment, showing loss of toxicity of pulverized root bark and leaflets in seven days or less, indicated the toxins are decomposed rapidly in soil. The present investigation, however, demonstrates the presence of toxicity for at least 13 days after addition of roots to soil. This suggests toxins were released continuously from the roots during the course of the experiment. Retardation of microbial activity by refrigeration undoubtedly also contributed. Thus, even though microbial decomposition is likely to be rapid under natural conditions, continuous toxin exudation by roots, or low temperatures, could maintain ecologically significant concentrations of toxins in the soil near roots.

One might argue that roots used in this investigation were different from intact roots attached to trees. Normal physiology may have been altered by excavation, detachment from trees, cutting, refrigeration, or other reasons, thereby increasing production or release of the toxins. This concern is valid

since production of secondary compounds sometimes increases in response to wounding or pathogens (Kemp and Burden, 1986; Cheniclet, 1987; Tuomi et al., 1988). Further investigations under more natural conditions are necessary. If toxins are released, we need to know how far and fast they move from the roots, how long they persist in the rhizosphere, and whether continuous exudation occurs and is sufficient to maintain a zone of toxicity around roots.

Toxicity of Field Soil near Ailanthus *Roots.* Growth of cress in soil collected adjacent to *Ailanthus* roots was less than in soil collected away from *Ailanthus* trees (Table 5). In the first bioassay, radicle growth was 85% of the control and not statistically significant. In the second, radicle growth was reduced to 77% and was significant. The two soils did not differ appreciably in pH, and only very slightly in moisture, after addition of deionized water.

This investigation provides further evidence that *Ailanthus* roots exude toxic material into nearby soil. The results are consistent with those of the previous experiment on excised root segments. Differences in pH or moisture are unlikely to account for the pattern since these were similar for the two soils. The reduced cress growth near *Ailanthus* roots could be due to nutrient depletion in the rhizosphere, but this is unlikely since the roots from which soil was sampled were comparatively large, lacked root hairs, and were not feeder roots. Release of toxin into the rhizosphere is, therefore, a valid possibility.

Biological Activity of Ailanthus *Stemflow.* Precipitation did not become toxic as it traveled along *Ailanthus* foliage, branches, and trunks. Instead, it became markedly stimulatory (Table 6). Stimulation ranged from 143% to 186% of the deionized water control for the five trees sampled. Control precipitation collected outside the *Ailanthus* grove was comparable to deionized water in effect on cress. Stemflow, therefore, does not appear to transport appreciable

TABLE 5. GROWTH OF GARDEN CRESS IN SOIL COLLECTED AWAY FROM, OR ADJACENT TO, *Ailanthus* ROOTS

Bioassay	Cress radicle length	Soil collection site: Away (control[a])	Soil collection site: Adjacent roots[b]
First	mm	35.1	29
	% of control	100	85
Second	mm	23.6	18.1[c]
	% of control	100	77[c]

[a] Moisture (after addition of 10 ml water/60 g soil) = 27%, pH = 4.7.
[b] Moisture (after addition of 10 ml water/60 g soil) = 33%, pH = 4.7.
[c] Significantly different from control in ANOVA at $P \leq 0.05$.

TABLE 6. EFFECT OF Ailanthus STEMFLOW AND CONTROL PRECIPITATION ON CRESS RADICLE GROWTH

	Controls				Ailanthus stemflow				
	Deionized water	Funnel A	Funnel B	Funnel C	Tree A	Tree B	Tree C	Tree D	Tree E
Cress radicle length[a]									
mm	20.8c	18.3c	19.7c	22.5c	29.9b	32.3ab	33.9ab	35.1ab	38.7a
% of control[b]	100c	88c	94c	108c	143b	155ab	163ab	168ab	186a
pH[c]	3.8	3.8	3.9	3.7	3.6	3.9	3.9	3.7	3.8

[a] Values followed by different letters differ at $P \leq 0.05$ in Duncan's multiple-range test.
[b] Data expressed as percentage of deionized water control.
[c] pH of deionized water control after adjustment with sulfuric acid; pH for rain and stemflow unadjusted.

amounts of toxin from the canopy or trunk to soil. The pH of all stemflow and control precipitation samples was between 3.6 and 3.9, indicating the strong influence of acidic deposition in the study area.

Several reasons could account for the stimulation caused by *Ailanthus* stemflow. Leaves, branch bark, and trunk bark contain phytotoxic material, but concentrations in the outer trunk bark are low compared to the inner bark (Heisey, 1990). The outer bark probably prevents sufficient amounts of toxin from being extracted by stemflow. Stimulation may indicate the presence of the allelochemicals at very low concentrations or the occurrence of other substances. Many such materials, including surface deposits of dust and airborne pollutants (Garten et al., 1988) or organic and inorganic constituents in foliage and bark (Tukey, 1969, 1971; Schier, 1987; Foster and Nicholson, 1988), could be dissolved as rain passes down the *Ailanthus* tree. Kozel and Tukey (1968) showed gibberellins can be leached from foliage and suggested these are involved in stimulatory effects of plant leachates.

Potential for Allelopathy under Natural Conditions. The present investigation and others (Mergen, 1959; Heisey, 1990) clearly demonstrate the occurrence of one or more phytotoxins in *Ailanthus* trees. The toxic material is strongly active against a number of plant species in vitro. On a dry weight basis, aqueous extracts of root bark are most toxic, but extracts of certain other plant parts also are active. Therefore, if allelopathy does occur under natural conditions, release of toxins from roots is more likely to be important than leaching from leaves or litterfall. The present results, showing greater inhibition by root bark and lack of phytotoxicity in stemflow, support this conclusion.

Concentrations of toxins in soil would be expected to be greatest near *Ailanthus* roots. The reduction of cress growth near detached *Ailanthus* roots in the laboratory indicates roots, especially small-diameter ones, do release toxins into adjacent soil. Soil collected near *Ailanthus* roots in the field supported less growth of cress than control soil. Further investigations on the quantity, duration, and spatial patterning of toxin release are necessary to adequately evaluate the significance of root exudation by *Ailanthus* in causing allelopathy.

Toxic material released by *Ailanthus* does not persist long in soil containing normal microflora. In most cases, the toxicity of root bark or leaflets disappeared within several days or weeks after the tissues had been incorporated into soil, suggesting microbial degradation. In the case of root bark, the initial inhibition was sometimes followed by stimulation after several days or weeks. These results indicate continued release of the allelochemicals from roots would be necessary to maintain toxicity in soil under natural conditions.

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NEW COMPONENTS IN DEFENSIVE SECRETION OF THE STRIPED SKUNK, *Mephitis mephitis*

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Abstract—GC-MS analysis of the anal sac secretion of the striped skunk (*Mephitis mephitis*), shows seven major components: (*E*)-2-butene-1-thiol, 3-methyl-1-butanethiol, *S*-(*E*)-2-butenyl thioacetate, *S*-3-methylbutanyl thioacetate, 2-methylquinoline, 2-quinolinemethanethiol, and *S*-2-quinolinemethyl thioacetate. The following compounds previously reported from this secretion could not be confirmed: bis(2-butenyl) sulfide, (*E*)-2-Butenyl methyl disulfide, (*E*)-2-butenyl propyl sulfide, butyl 3-methylbutyl disulfide, and 2-butenyl butyl disulfide.

Key Words—*Mephitis mephitis*, striped skunk, anal sac secretion, (*E*)-2-butene-1-thiol, 3-methyl-1-butanethiol, *S*-(*E*)-2-butenyl thioacetate, *S*-3-methylbutanyl thioacetate, 2-methylquinoline, 2-quinolinemethanethiol, *S*-2-quinolinemethyl thioacetate, (*E*)-2-butenyl methyl disulfide, (*E*)-2-butenyl propyl sulfide, butyl 3-methylbutyl disulfide, 2-butenyl butyl disulfide, bis(2-butenyl) sulfide.

INTRODUCTION

The chemical analysis of the anal sac secretion from the striped skunk, *Mephitis mephitis*, has been the subject of many previous studies. Aldrich (1896) identified 1-butanethiol as a major component of this malodorous secretion, and a study by Aldrich and Jones (1897) identified 2-methylquinoline (I) (Figure 1). Stevens (1945) found it to contain more than 2% dicrotyl sulfide [bis(2-butenyl) sulfide, II]. Andersen and Bernstein (1975) reported (*E*)-2-butene-1-thiol (III), 3-methyl-1-butanethiol (IV), and (*E*)-2-butenyl methyl disulfide (V) as the major components of this secretion using NMR and gas chromatography and showed 1-butanethiol was not a major component. A subsequent study by Andersen and

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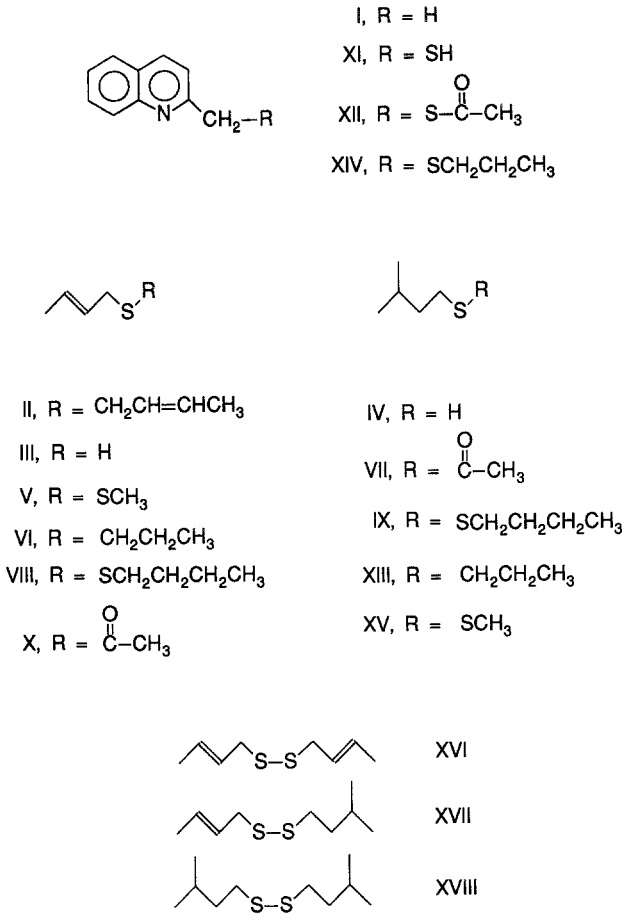


FIG. 1. Structures of compounds discussed in article.

coworkers used gas chromatography–mass spectrometry (GC-MS) to investigate the minor components of this defensive secretion (Andersen et al., 1982). In this latter study, Andersen and coworkers were unable to confirm the presence of (*E*)-2-butenyl methyl disulfide, but (*E*)-2-butenyl propyl sulfide (VI) and *S*-3-methylbutyl thioacetate (VII) were reported at 7% and 1.4% of the volatiles, respectively. They confirmed Stevens' identification of dicrotyl sulfide [bis(2-butenyl)sulfide], finding 1.1% of this compound. Furthermore, three disulfides, 2-butenyl butyl disulfide (VIII), butyl 3-methylbutyl disulfide (IX), and an isomer of butyl 3-methylbutyl disulfide, were reported to make up 10.7% of the volatiles.

In the present study, using GC-MS, seven components were found to make up the bulk of the volatiles in this secretion, including three that had not pre-

viously been reported. These new compounds are *S*-(*E*)-2-butenyl thioacetate (X), 2-quinolinemethanethiol (XI), and *S*-2-quinolinemethyl thioacetate (XII). A number of compounds previously identified could not be confirmed. These are (*E*)-2-butenyl methyl disulfide, (*E*)-2-butenyl propyl sulfide, bis(2-butenyl) sulfide, and the three disulfides previously identified by GC-MS (VIII, IX, and the isomer of IX).

Several other compounds were prepared during the current study. 3-Methylbutyl propyl sulfide (XIII) and propyl 2-quinolinemethyl sulfide (XIV), compounds with the same molecular weights as compounds VII and XII, were prepared since Andersen and coworkers had identified a propyl sulfide derivative (VI) from this secretion. 3-Methylbutyl methyl disulfide (XV) was prepared as a by-product in the synthesis of compound V from anal gland secretion. Bis[(*E*)-2-butenyl] disulfide (XVI), (*E*)-2-butenyl 3-methylbutyl disulfide (XVII), and bis(3-methylbutyl) disulfide (XVIII) were prepared to show disulfides VIII and IX were not destroyed during analysis.

METHODS AND MATERIALS

All skunks were from Humboldt County, California, and were obtained alive from a professional trapper who removed them from areas where they were a nuisance. Two were male and two were female. They were sacrificed with diethyl ether and within an hour a sample of the defensive secretion was obtained by inserting a needle into the anal sac and withdrawing the contents. A portion of this secretion was immediately placed in diethyl ether and analyzed by GC-MS.

GC-MS was done on a Hewlett Packard gas chromatograph (model 5890) fitted with a mass selective detector (model 5970) using a 12-m cross-linked methyl silicone capillary column. The gas chromatograph was programmed so the oven temperature was kept at 40°C for 4 min, then increased to a final temperature of 250°C at a rate of 30°C/min and kept at this temperature for 4 min. Mass spectral fragments below $m/z = 35$ were not recorded. An isotope peak ($M^+ + 2$) was observed for all sulfur-containing compounds that had a molecular ion. The relative amount of each component is reported as the percent of the total ion current.

FT-IR was done on a Perkin-Elmer 1600 and NMR on a Perkin-Elmer R12A using TMS as an internal standard.

3-Methyl-1-butanethiol, 2-methylquinoline, and 1-chloro-2-butene were purchased from Aldrich Chemical Co. 2-Bromomethylquinoline was prepared from 2-methylquinoline by conversion to 2-tribromomethylquinoline (Hammick, 1923) and subsequent reduction with freshly prepared tin(II) bromide (Brown et al., 1951).

(E)-*2-Butenyl methyl disulfide (V) and 3-methylbutyl methyl disulfide (XV).* To 5 mg of skunk anal sac secretion (source of III and IV) in 50 ml of Et_2O at 0°C was added 1.0 ml of methanethiol and 100 mg of I_2. The solution was stirred for 15 min at 0°C. It was washed with 25 ml of H_2O and dried (Na_2SO_4). GC-MS analysis of this solution showed three compounds that were not in the original solution: dimethyl disulfide (*RT* = 2.00 min, M^+ at *m/z* = 94); compound V at 6.62 min, EI-MS, *m/z* = 134(M^+, 18), 80(13), 79(6), 64(6), 55(100), 53(9), 47(6), 45(18), 43(10), and 39(20); and compound XV at 7.10 min, EI-MS, *m/z* = 152(2), 150(M^+, 18), 80(11), 79(6), 71(12), 55(8), 45(12), 43(100), 41(18), and 39(9).

S-(E)-*2-Butenyl propyl sulfide (VI).* To 11.3 g (0.1 mol) of 1-chloro-2-butene (80%, predominantly the *trans* isomer) in 25 ml of MeOH was added 7.6 g (0.1 mol) of thiourea. After refluxing for 16 hr, 8.0 g (0.2 mol) of NaOH was added over 10 min and refluxing was continued for 1 hr. 1-Bromopropane (24.6 g, 0.2 mol) was added dropwise and then refluxed for 1 hr. The reaction mixture was added to 100 ml of Et_2O and the solids removed by filtration. The liquid was washed with 2 × 25 ml of 5% $NaHCO_3$, dried (Na_2SO_4), and concentrated in vacuo. Yield 7.0 g. The spectral properties of this liquid are 60 MHz [^{1}H]NMR (CCl_4) δ = 5.4 (m, 2H), 2.95(d, 2H), 2.3(t, 2H), 1.65(d, 3H), 1.45(m, 2H), and 0.95(t, 3H); FT-IR (neat) 3020, 2962, 2934, 2872, 1649, 1454, 1377, 1224, 1094, and 964 cm^{-1}; and EI-MS *m/z* = 130(M^+, 25), 88(7), 87(13), 55(100), 54(35), 53(13), 47(13), 45(18), 41(18), and 39(23).

S-*3-Methylbutyl thioacetate (VII).* To 1.04g (10 mmol) of 3-methyl-1-butanethiol in 50 ml of 95% EtOH was added 0.40 g (10 mmol) of NaOH. The mixture was stirred on a steam bath until the NaOH had dissolved, then cooled in an ice bath, and 2.4 g (30 mmol) of acetyl chloride was added dropwise to the stirred solution. The solution was stirred for 15 min and then the solvent was removed in vacuo. The residue was extracted with 50 ml of Et_2O. This solution was washed with 2 × 25 ml of 5% $NaHCO_3$, dried (Na_2SO_4), filtered, and evaporated to dryness. Yield 0.2 g. EI-MS, *m/z* = 146(M^+, 0.4), 103(2), 86(12), 70(13), 69(6), 61(5), 55(8), 47(3), 43(100), 41(11), and 39(6).

S-(E)-*2-Butenyl thioacetate (X).* To 11.3 g (0.1 mol) of 1-chloro-2-butene (80%, predominantly the *trans* isomer) in 25 ml of MeOH was added 7.6 g (0.1 mol) of thiourea. After gently refluxing for 16 hr, 8.0 g (0.2 mol) of NaOH was added over 10 min and refluxing was continued for 1 hr. The mixture was placed in an ice bath and was stirred while 15.7 g (0.20 mol) of acetyl chloride was added dropwise over 15 min. The mixture was stirred for 30 min and then concentrated in vacuo. The residue was extracted with 2 × 50 ml of Et_2O. This solution was washed with 5% $NaHCO_3$, dried (Na_2SO_4), and concentrated in vacuo. Yield 3.5 g. The spectral properties of this liquid are 60 MHz [^{1}H]NMR (CCl_4) δ = 5.45(m, 2H), 3.35(d, 2H), 2.2(s, 3H), and 1.65(d, 3H); FT-IR (neat) 3023, 2965, 2937, 2917, 1694, 1451, 1354, 1228, 1133, and 963 cm^{-1};

and EI-MS m/z = 130 (M^+, 10), 88(21), 87(9), 59(5), 55(34), 54(10), 53(9), 50(17), 43(100), and 39(14).

2-Quinolinemethanethiol (XI). To 3.6 g (0.016 mol) of 2-bromomethylquinoline in 50 ml of 95% ethanol was added 2.3 g (0.03 mol) of thiourea. The mixture was refluxed 3 hr, cooled, and the solvent removed in vacuo to give an oil. To the oil was added 50 ml of H_2O and 2.0 g (0.05 mol) of NaOH. This was refluxed for 3 hr, cooled, and washed with 3 × 25 ml of Et_2O. The aqueous solution was carefully neutralized to pH 7 with 6 M HCl and then extracted with 3 × 25 ml of Et_2O. The Et_2O was removed in vacuo to give 2.0 g of product. EI-MS, m/z = 176(11), 175(M^+, 100), 174(71), 142(10), 130(37), 129(55), 128(26), 116(11), 115(25), and 102(11).

S-*2-Quinolinemethyl thioacetate (XII).* To 175 mg (1.0 mmol) of X in 10 ml of 95% EtOH was added 40 mg (1.0 mmol) of NaOH, and the solution was refluxed until the NaOH had dissolved. The solution was cooled to room temperature, and 393 mg (5.0 mmol) of acetyl chloride was added dropwise and stirred for 15 min. The solvent was removed in vacuo and the residue extracted with 10 ml of Et_2O. After being washed with 5 ml of 5% $NaHCO_3$, the Et_2O solution was dried (Na_2SO_4), and the solvent removed in vacuo. Yield 94 mg. EI-MS, m/z = 217(M^+, 3), 176(10), 175(55), 174(100), 142(8), 130(14), 129(20), 128(20), 115(12), and 43(48).

3-Methylbutyl propyl sulfide (XIII). To 1.04 g (10 mmol) of 3-methyl-1-butanethiol in 50 ml of 95% EtOH was added 0.40 g (10 mmol) of NaOH. The mixture was refluxed until the NaOH had dissolved and then 6.2 g (50 mmol) of 1-bromopropane was added dropwise over several minutes. The solution was gently refluxed for 1 hr, cooled, and solvent removed in vacuo. The residue was extracted with 50 ml of Et_2O. This solution was dried (Na_2SO_4), filtered, and evaporated to dryness. Yield 0.4 g. EI-MS, m/z = 146(M^+, 26), 103(10), 89(12), 70(100), 61(37), 55(50), 47(21), 43(48), 42(34), and 41(48).

Propyl 2-quinolinemethyl sulfide (XIV). To 175 mg (1.0 mmol) of XI in 10 ml of 95% EtOH was added 40 mg (1.0 mmol) of NaOH, and the solution was refluxed until the NaOH had dissolved. Then 1.23 g (10.0 mmol) of 1-bromopropane was added and the mixture refluxed for 1 hr. On cooling, the volatiles were removed in vacuo, and the residue was extracted with 10 ml of Et_2O. After being washed with 5 ml of 5% $NaHCO_3$, the Et_2O solution was dried (Na_2SO_4), and the solvent removed in vacuo. Yield 180 mg. EI-MS m/z = 174(3), 144(9), 143(100), 140(4), 129(3), 128(7), 116(6), 115(9), 101(3), and 89(4).

Bis[(E)*-2-butenyl*] *disulfide (XVI),* (E)*-2-Butenyl 3-methylbutyl disulfide (XVII), and Bis(3-methylbutyl) disulfide (XVIII).* To 5 mg of skunk anal sac secretion in 50 ml of Et_2O was added 100 mg of I_2. After being stirred for 15 min, the solution was washed with 25 ml of H_2O and dried (Na_2SO_4). GC-MS analysis showed the thiols (III and IV) had completely reacted to give the fol-

lowing disulfides: compound XVI at 8.42 min, EI-MS m/z = 174(M^+, 5), 128(2), 122(2), 120(15), 56(4), 55(100), 53(7), 46(1), 45(10), and 38(12); compound XVII at 8.71 min, EI-MS m/z = 190(M^+, 7), 136(12), 71(10), 56(4), 55(100), 53(6), 45(7), 43(30), 41(14), and 39(12); and compound XVIII at 8.97 min, EI-MS m/z = 207(2), 206(M^+, 13), 136(4), 103(3), 102(3), 71(35), 55(11), 43(100), 41(19), and 39(8).

(*E*)-2-Butene-1-thiol from the skunk's secretion has the following mass spectrum; EI-MS m/z = 88(37), 73(6), 55(100), 54(42), 53(25), 51(8), 50(8), 47(13), 45(35), and 39(52).

RESULTS AND DISCUSSION

GC-MS analysis of a diethyl ether solution of the anal sac secretion from four skunks showed seven volatile compounds at a concentration greater than 1%, that totaled 96–98% of the volatiles. These compounds and their amounts (maximum variation) are (*E*)-2-butene-1-thiol (38–44%), 3-methyl-1-butanethiol (18–26%), (*S*)-(*E*)-2-butenyl thioacetate (12–18%), (*S*)-3-methylbutanyl thioacetate (2–3%), 2-methylquinoline (4–11%), 2-quinolinemethanethiol (3–12%), and (*S*)-2-quinolinemethyl thioacetate (1–4%). The identification of these compounds is discussed in order of their gas chromatographic retention time (*RT*).

(*E*)-2-Butene-1-thiol (*RT* = 1.73 min) and 3-methyl-1-butanethiol (*RT* = 2.62 min) were reported previously from this secretion. (*E*)-2-Butene-1-thiol was identified by its mass spectrum. 3-Methyl-1-butanethiol from the secretion had the same retention time and mass spectrum as an authentic sample.

The third most abundant component (*RT* = 6.16 min) was identified as *S*-(*E*)-2-butenyl thioacetate. The mass spectrum of this compound has a molecular ion at m/z = 130 and a base peak at m/z = 43, indicating it may contain an acetyl group (CH_3CO-) attached to a thiobutenyl group. A possible candidate for this compound, *S*-(*E*)-2-butenyl thioacetate, was prepared and had an identical retention time and mass spectrum as the natural compound. A strong FT-IR absorption at 963 cm^{-1} indicated a *trans* geometry for the double bond. This component appears to have been identified previously as (*E*)-2-butenyl propyl sulfide, a compound with the same molecular weight and similar GC retention time as *S*-(*E*)-2-butenyl thioacetate (Andersen et al., 1982). A synthetic sample of this sulfide had a different retention time (6.29 min) and a different mass spectrum from the compound in the secretion.

It is also likely that *S*-(*E*)-2-butenyl thioacetate has previously been identified as (*E*)-2-butenyl methyl disulfide (Andersen and Bernstein, 1975). The disulfide was identified by comparison to the GC retention time and NMR spectrum of a synthetic sample. The reported NMR spectrum of the disulfide is

almost identical to the spectrum of *S*-(*E*)-2-butenyl thioacetate. When (*E*)-2-butenyl methyl disulfide was not found during one investigation, it was suggested that "it does not survive the high injector temperature" (Andersen et al., 1982). To check this, a sample of this disulfide was prepared by treating the anal sac secretion (source of III and IV) with methanethiol and iodine in an ether solution cooled to 0°C. GC-MS analysis of the reaction mixture showed three compounds not found in the skunk's secretion. These were identified as dimethyl disulfide (*RT* = 2.00 min), (*E*)-2-butenyl methyl disulfide (*RT* = 6.62 min), and 3-methylbutyl methyl disulfide (*RT* = 7.10 min) by their mass spectra. Thus, it is clear that (*E*)-2-butenyl methyl disulfide is not a major component in this secretion.

S-3-Methylbutyl thioacetate (*RT* = 6.56 min) was identified previously by comparison to a spectrum in the NBS Library (Andersen et al., 1982). A synthetic sample of this compound had an identical mass spectrum and retention time to that found in the skunk secretion. 3-Methylbutyl propyl sulfide, a compound with the same molecular weight that might be expected to give a similar mass spectrum was prepared. It had a different retention time (6.77 min) and mass spectrum.

2-Methylquinoline (*RT* = 8.35 min) has been previously reported from this secretion and was identified by comparison to an authentic sample.

The sixth compound had a retention time of 9.85 min and an odd mass molecular ion (base peak) at *m/z* = 175, suggesting that it contained a nitrogen. Analysis of the spectrum showed fragments from the loss of SH ($M^+ - 33$) and a CH_2S ($M^+ - 46$). Moreover, it had a fragmentation pattern similar to 2-methylquinoline. This suggested it might be 2-quinolinemethanethiol. A synthetic sample of this compound was prepared from 2-bromomethylquinoline and thiourea followed by basic hydrolysis. It had the same retention time and mass spectrum as the natural compound. This compound previously has been prepared by other methods (Yousif et al., 1982; Sturis et al., 1985).

Finally, the last compound had a retention time of 10.89 min. The mass spectrum had a weak molecular ion at *m/z* = 217, a base peak at *m/z* = 174 (loss of 43 amu), and a fragmentation pattern similar to 2-quinolinemethanethiol, suggesting this compound is either an *S*-acetyl or *S*-propyl derivative of 2-quinolinemethanethiol. Both compounds were prepared. The acetate derivative, *S*-2-quinolinemethyl thioacetate, had identical GC-MS properties to the compound found in the secretion. The retention time (10.76 min) and mass spectrum of the propyl derivative, propyl 2-quinolinemethyl sulfide, were different.

Bis(2-butenyl) sulfide, reported by Stevens (1945) to make up more than 2% of the secretion and reconfirmed by Andersen and coworkers (1982) at 1.1%, was not observed in fresh secretion. An examination of all minor components in the secretion failed to find a compound with a molecular ion at *m/z* = 142.

This compound should have survived the analysis, as (*E*)-2-butenyl propyl sulfide was easily detected. The three butyl-substituted disulfides (VIII, IX, and an isomer of IX) reported in the GC-MS study of Andersen et al. (1982) also were not seen in the present study. To check if disulfides similar to VIII and IX might be destroyed during the GC-MS analysis, a sample of skunk's secretion in diethyl ether was oxidized with I_2. GC-MS analysis showed the thiols (III and IV) had reacted completely to give three disulfides, bis [(*E*)-2-butenyl] disulfide (*RT* = 8.41 min), (*E*)-2-butenyl 3-methylbutyl disulfide (*RT* = 8.71 min), and bis(3-methylbutyl) disulfide (*RT* = 8.97 min). Thus, the butyl-substituted disulfides would have been detected in the present study. [(*E*)-2-Butenyl 3-methylbutyl disulfide was observed as a minor component (0.2–1.6%) in all of the samples.]

There may be several explanations why compounds previously identified could not be confirmed in this study. Some may be artifacts caused by bacterial action, air oxidation, or other factors during the isolation procedures. Stevens identified bis(2-butenyl) sulfide from secretion that had been stored for several months, treated with ethanolic mercuric chloride, and distilled three times. When Andersen and coworkers confirmed the identification of this compound and identified the disulfides (VIII and IX), they used a stored solution that was distilled (80°C, 0.2 mm) before analysis. Alternatively, the defensive secretion of skunks in New England may differ from those of skunks in northern California.

In conclusion, with the exception of 2-methylquinoline, the major volatile components of the striped skunk's defensive secretion identified in this study exist as thiols or as acetate derivatives of these thiols. Possibly, they are biosynthesized as the thoiacetates and then hydrolyzed to the thiol, since thioacetates are used extensively in many biosynthetic pathways.

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GERANYLLINALOOL (DITERPENE ALCOHOL) An Insecticidal Component of Pine Wood and Termites (Isoptera: Rhinotermitidae) in Four European Ecosystems

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Abstract—Geranyllinalool is an insecticidal component of pine wood and of the defensive secretion of *Reticulitermes* species. Lethal doses (LD_{50}) were calculated for termites and various ant species. Termite workers were very resistant (10,000 ppm). Ant resistance varies among species according to their trophic strategy and attack mode. The geranyllinalool acts as a natural insecticide against some ant predators (LD_{50} = 6 ppm), which use raid or chemical crypsis to invade termite nests. In contrast, predators laying the venom on the cuticle of their termite prey were very resistant (10,000 ppm). Generally, ants acting as space competitors could detoxify the quantity emitted by one termite soldier (18 ppm).

Key Words—Geranyllinalool, toxicity, Isoptera, Rhinotermitidae, *Reticulitermes*, soldier, *Pinus*, ants, predators, competitors.

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INTRODUCTION

Many investigations on cephalic secretions of termites and secondary compounds of wood have stressed the elucidation of the chemistry of these defensive compounds without testing their effects on natural predators or competitors. Three *Reticulitermes* species (Isoptera: Rhinotermitidae), common in Europe, live in trunks and logs of *Pinus* wood (Clément, 1977). Two species *R. santonensis* and *R. (lucifugus)* grassei are sympatric in Charente maritime; the third, *R. (lucifugus) banyulensis*, is allopatric to the others in a Mediterranean area.

Relationships between ants and termites were discribed by Guille-Escuret (1981) and Lemaire (1982) in the Atlantic ecosystem of decaying pine wood. Ants can be divided into two groups in their relations with termites: as competitors for space distribution and as predators. The main families of ant predators are Ponerinae (Weber, 1949; Longhurst, 1978; Wheeler, 1936), Pheidolae (Sjöstedt, 1907), and some Formicinae (Mathur, 1962; Weber, 1964; Blake, 1941; Sheppe, 1970).

Termite soldier defensive secretions are composed variously of monoterpenes, sesquiterpenes, diterpenes, and small alkanes and alkenes (for review see Blum, 1981; and Prestwich, 1973, 1984). Many species synthesize terpenes, but repellency and toxicity of these compounds have been analyzed in only a few cases and on nontermite predators. Only one author (Mill, 1983) reports that total secretions of the frontal gland of various termite soldiers are toxic to two predatory ants. Howse (1975) and Coles de Negret and Howse (1983) noted only narcotic effects on ants of monoterpenes emitted by termites of *Nasutitermes* sp. A behavioral modification of two Ponerines (*Odontomachus* sp. and *Megaponera foetens*) is induced by sesquiterpenoids (Evans et al., 1977). Hrdy et al. (1977) published toxicity of a diterpene secreted by *Nasutitermes* sp. soldiers on *Musca domestica* (21.9 μg for one female; i.e., 270,000 ppm).

In Europe, the major compound in the secretion of the frontal gland of soldiers of the genus *Reticulitermes* is a diterpene alcohol: geranyllinalool. The average quantities of geranyllinalool emitted by termite soldiers are species-specific and show geographic variation (Parton et al., 1981; Baker et al., 1982; Lemaire, 1990). This diterpene alcohol also was found in plants such as *Helychrysum* (Bohlman and Suwita, 1979) *Gregeiria* (Bohlman et al., 1982), *Picea* (Schmidt and Pentegova, 1977), and *Jasmine* (Verzele et al., 1981). Juettner and Bogenschuetz (1983) suggested it had an antifungal action in association with other terpenes. One of its derivatives, geranyl-geraniol, acts as an anti-chlorophyll synthetase (Ruediger et al., 1980).

The presence of geranyllinalool in both wood and defensive compounds emitted by xylophagous insects suggests the need for an accurate analysis of its

toxicity against both xylophagous insects and their predators. This paper reports our tests of the toxicity of geranyllinalool against pine-inhabiting termites and their ant predators and competitors in four French ecosystems; it determines the relationships between geographic variation of quantity of the diterpene alcohol in the soldiers' defensive secretion in the presence of local predators and competitors, and examines the influence of selective pressure on defensive compounds and detoxification efficiency.

METHODS AND MATERIALS

Insect Collection. Portions of termite societies of the three species were collected from fallen logs or tree trunks in the four ecosystems (Figure 1). The first ecosystem is an Atlantic area (LC, Charente-Maritime) where *Reticulitermes santonensis* and *R. (l.) grassei* (Rg1) are sympatric. The second one is near Bordeaux (AR) where only *R. (l.) grassei* (Rg1) is present. Forests in both areas are composed of *Pinus pinaster* and have humid and temperate climate. The third ecosystem is in a continental forest (EY) with Mediterranean influences, composed of mixed deciduous and coniferous species where *R. (l.) grassei* populations are living (Rg2). The fourth area (BA) is in a typical Mediterranean ecosystem where *R. (l.) banyulensis* is common (Clément, 1977).

Workers and soldiers were used for testing because they belong to permanent castes. Soldiers were dipped in pentane for chemical analysis of the defensive compounds. To determine the amount of geranyllinalool emitted in a drop of secretion, a microcap was used to collect the mixture of defensive compounds during an attack (One microcap per soldier; three replicates per site of collection). Workers were placed in Petri dishes in groups of 10.

Portions of ant nests were collected near termite nests. Assays were generally run immediately after collection.

Chemistry. Secondary compounds from decaying wood (two to three years of decay) were extracted in pentane after 30 min crushing–sonication and concentrated under a nitrogen stream. Gas chromatographic analyses of wood and soldiers extracts were performed on a Delsi 300 gas chromatograph (Sté. Delsi-Nermag, Argenteuil, France) equipped with a FID detector. A 25-m × 0.25-mm-ID capillary column packed with CPSil5 (Chrompack, The Netherlands) was used to separate the compounds. The oven was programmed from 30°C to 300°C at 3°C/mn. Mass spectra of geranyllinalool were obtained using a Nermag 10-10C at an ionizing voltage of 70 eV coupled with a Sidar data processing system. The gas chromatograph was equipped with the same capillary column. Synthetic geranyllinalool with four isomers from BASF Inc. was used to compare natural diterpene retention time on two capillary columns (CPSil5, non polar, and CPWax51, polar; from Chrompack Inc.) and mass spectra.

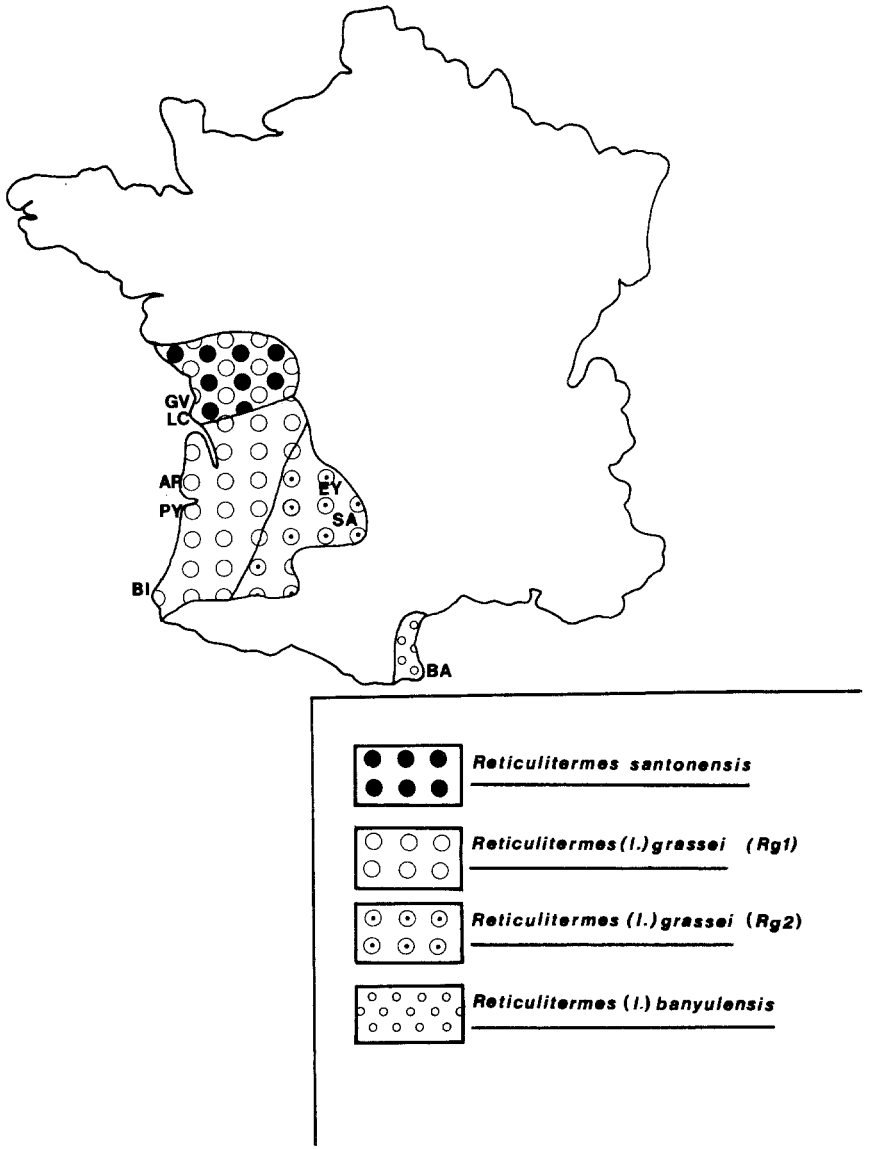


FIG. 1. Geographical distribution of the three termite species in France. *R. santonensis* and *R. (l.) grassei* (Rg1) are sympatric in the first ecosystem (GV and LC sites) while *R. (l.) grassei* (Rg1) is the only species in the second Atlantic ecosystem (AR, PY and BY sites). *R. (l.) grassei* termites are noted Rg2 in the continental ecosystem (EY and SA sites) and *R. (l.) banyulensis* is allopatric with the two other species in the mediterranean ecosystem (BA site).

N-Dodecane and 2-nonanal were used as external standards for geranyllinalool quantification with an Enica computer.

Toxicology. Toxicity of geranyllinalool was quantified in the three species of *Reticulitermes*, 12 species of ant predators and competitors (Table 1), and one orthopteran, *Schistocerca gregaria*. The last was used to compare with commercial insecticides. We used termite workers (fifth to eighth instars) within 15 days after field collection. Fifth-instar larvae of *Schistocerca gregaria* were used two days after molting.

A preliminary screening was carried out to assay solvent toxicity on each insect species. Generally, pentane was the least toxic solvent, except for *Monomorium* sp., for which dichloromethane was the only nontoxic solvent. Probit analyses were used between doses $1–10^6$ ng/insect. One microliter of solution in pentane or dichloromethane was applied topically on the abdomen of an insect immobilized on ice in groups of 10 termites or ants for each concentration with five replicates; one group of 10 *Schistocerca* was also tested. After 15 hr in 60-mm-diameter Petri dishes on moist filter paper in the dark (25°C), mortality was noted and probit analyses were undertaken by computer to determine LD_{50} with its Standard error (Hewlett-Packard 85, Programme J.L. Clément, J.C. Baehr, P. Porcheron).

TABLE 1. RELATIONSHIPS BETWEEN ANTS AND *Reticulitermes* SPECIES IN FOUR ECOSYSTEMS

	Termites			
	R.(l.)g.			
Ants	Rg1	Rg2	R.s.	R.(l.)b.
Predators				
Pheidole pallidula		X		X
Hypoponera eduardi	X		X	
Leptothorax unifasciatus	X	X	X	
Leptothorax nylanderi	X	X	X	
Leptothorax parvulus	X	X	X	
Myrmica rugulosa	X	X	X	
Aphaenogaster subterranea	X	X	X	X
Crematogaster scutellaris				X
Monomorium sp.	X		X	
Competitors				
Leptothorax lichtensteni				X
Leptothorax racovitzae				X
Lasius emarginatus	X	X	X	

Ecology. Ants were classified into two groups relative to termites: predators for which predatory behavior under natural conditions had been observed (attack of termite nests or part of termite body in rubbish mounds) and competitors for space with termites inside the same *Pinus* trunks.

RESULTS

Dosage. Geranyllinalool constitutes 10 ppm of total weight of decaying *Pinus pinaster.* Contrary to analyses by Parton et al. (1981) using packed columns, geranyllinalool was found in the soldier frontal gland of all three species (Table 2), including *R. santonensis* (30–100 ng in each frontal gland reservoir; 20–80% of the total secretion). Soldiers of *R. (l). grassei* emit 800–7000 ng in the Atlantic area—Rg1— and 600–20,000 ng of geranyllinalool in the continental area—Rg2 (80–99% of the total secretion for both populations). Soldiers of *R. (l.) banyulensis* emit 200–1500 ng (95–100% of the total secretion). The volume of the drop emitted during agressive encounters is only 10% of the total quantity of the reservoir. The natural isomer of geranyllinalool had the same retention time as the fourth BASF isomer on a nonpolar capillary column.

Insect Resistance. The average LD_{50} of the 16 insects allowed us to distinguish four groups (Table 3) based both on the status of the ants and their resistance:

In group A, LD_{50} is near 7 ppm. This group is composed of two predatory ant species, one in the south and one in the northwest of France: *Pheidole pallidula* attacking *R. (l.) banyulensis* and *R. (l.) grassei* (Rg2); *Hypoponera eduardi* attacking *R. santonensis* and *R. (l.) grassei* (Rg1).

In group B, LD_{50} are between 5 and 20 ppm. This group is composed of two Mediterranean competitors of *R. (l.) banyulensis* (*Leptothorax lichtensteni* and *L. racovitzae*).

In group C, the LD_{50}s are between 100 and 600 ppm. Only ant predators are included in this group with predominance of ants from the southwest area. The doses corespond to high quantities of the geranyllinalool emitted in one drop by termite soldiers (2000 ng).

In group D: LD_{50}s are between 3000 ng and 11000 ppm. A termite competitor (*Lasius emarginatus*) and a strict predator (*Leptothorax parvulus*) are included in this range. *Schistocerca gregaria* (Orthoptera) has an LD_{50} of 3500 ppm. The three termite species show a great resistance to geranyllinalool. *R. (l.) banyulensis* is the less susceptible species with an LD_{50} of 11,000 ppm. We must note that only soldiers of this species synthesize this diterpene (Lemaire, 1990).

TABLE 2. DOSAGE OF GERANYLLINALOOL IN SECRETION OF FRONTAL GLAND OF *Reticulitermes* SOLDIERS (COLLECTED BY MICROCAPS)

Society	Site of collection	Mean quantity of geranyllinalool (nanogram per soldier)
R. santonensis		
GV1	Oléron	63
GV2	Oléron	31
GV3	Oléron	81
LC1	La Coubre	100
LC2	La Coubre	100
LC3	La Coubre	32
R. (l.) grassei		
EY1	Les Eyzies	2290
EY2	Les Eyzies	6900
EY3	Les Eyzies	3750
SA1	Sarlat	5355
SA2	Sarlat	18450
SA3	Sarlat	4820
SA4	Saint-Chamassy	600
SA5	Saint-Chamassy	800
PY	Pylat	800
AR	Arcachon	1080
BI1	Biarritz	2130
BI2	Biarritz	1720
LC4	La Coubre	6290
LC5	La Coubre	4565
LC6	La Coubre	6710
R. (l.) banyulensis		
BA1	Tautavel	200
BA2	Tautavel	250
BA3	Vingrau	400
BA4	Vingrau	280
BA5	Banyuls	1050
BA6	Banyuls	250
BA7	Banyuls	1420

DISCUSSION

Termite Resistance to Geranyllinalool. Termite workers show the greatest resistance to this natural compound among the insects of the four ecosystems. Nevertheless, termite soldiers are more resistant than workers of their own species, seven times in *R. santonensis* and 16 times in *R. (l.) grassei* (Nagnan,

TABLE 3. LD_{50} OF GERANYLLINALOOL ON TERMITES AND ITS ANT PREDATORS AND COMPETITORS

Group	Insect species in *Pinus* wood in four ecosystems	LD_{50} for one individual (nanogram ± SD)	LD_{50} (ppm ± SD)
A	*Pheidole pallidula*	1.8 (0.2)	7.2 (0.8)
	Hypoponera eduardi	3.1 (1)	7.3 (2.5)
B	*Leptothorax lichtensteni*	3.6 (0.6)	5.9 (1)
	Leptothorax racovitzae	12.7 (3.4)	18.1 (4.8)
C	*Leptothorax unifaciatus*	100 (15)	160 (20)
	Leptothorax nylanderi	100 (15)	180 (20)
	Myrmica rugulosa	580 (260)	390 (170)
	Aphaenogaster subterranea	870 (360)	360 (150)
	Crematogaster scutellaris	1650 (800)	610 (270)
D	*Reticulitermes (l.) grassei*	7400 (700)	2740 (250)
	Lasius emarginatus	4870 (1700)	3250 (1200)
	Reticulitermes santonensis	11100 (2200)	4480 (800)
	Leptothorax parvulus	5520 (2000)	9360 (3500)
	Monomorium sp.	1700 (500)	9440 (2800)
	Reticulitermes (l.) banyulensis	20200 (110)	10600 (60)

1989). Two major barriers could prevent the toxic effects of geranyllinalool: the epicuticular waxes, acting as a chemical protection against toxin penetration, and biochemical detoxification in the hemolymph by enzymatic action. The epicuticular waxes of *Reticulitermes* are composed of n-alkanes, methylalkanes, and alkenes (Howard et al., 1978, 1982; Howard, 1982; Clément and Lange, 1984) but in lower quantities (110 ppm for *R. santonensis*, 80 ppm for *R. (l.) grassei* and 120 ppm for *R. (l.) banyulensis*; Bagnères, 1989) than for ants (260 ppm for *Leptothorax nylanderi* and 700 ppm for *Hypoponera eduardi*). So we must conclude that biochemical detoxification mechanisms reduce termite mortality, as has been found by Spanton and Prestwich (1981, 1982) for *Prorhinotermes simplex* or *Schedorhinotermes lamanianus*.

Termite workers are resistant to normal doses emitted by a soldier (2 μg in a drop). Their detoxification mechanism also allows them to invade *Pinus* trunks without lignivorous ants competing at the beginning of wood degradation, because we showed elsewhere that oral toxicity does not act in wood feeding (Nagnan, 1988). Large *R. (l.) grassei* and *R. (l.) banyulensis* populations living in warm and dry areas have a high diterpene alcohol percentage in the total secretion emitted by a soldier. The climatic conditions could explain the presence of nontoxic monoterpenes in the soldiers secretion of the Atlantic area (Lemaire et al., unpublished results). We can consider that these compounds act as a solvent for the diterpene alcohol, which is solid under these temperature

conditions (15°C). In contrast, the average temperature in the Mediterranean area is about 20°C, and geranyllinalool is in the liquid phase. Thus, the nontoxic monoterpenes would not be necessary in the defensive secretion of soldiers of *R. (l.) banyulensis*.

Ant Resistance to Geranyllinalool. Differences between species appear to be correlated with differences in their trophic behavior. Except for *Lasius emarginatus*, competitors are very sensitive to the defensive compounds of termites (group B). The strict predators are able to detoxify the diterpene alcohol up to the general quantity emitted in the range of the drop secretion. Ants applying their venom on prey cuticle [*Monomorium* for *R. santonensis* and *R. (l.) grassei*, *Crematogaster* for *R. (l.) banyulensis*], are more resistant than the stinging ants (*Leptothorax* spp., *Myrmica*, and *Aphaenogaster*). These two species use a raid strategy and need a breach in the wood protecting the nest. The great differences in detoxification abilities in the genus *Leptothorax* are more linked to the same trophic status (predator/competitor) than to their phylogenetic proximity (*L. lichtensteni*, *L. parvulus* and *L. nylanderi*, for example). Geranyllinalool is extremely toxic to two predatory ant species, but they use two strategies to minimize this disadvantage: (1) *Hypoponera eduardi* in Europe, similar to *Ponera pensylvatica* in the United States (Lemaire, 1985), uses a chemical crypsis that allows it to move inside *Reticulitermes* nests without termite detection. Ethological and chemical analyses show that cuticular hydrocarbons are responsible for the chemical crypsis. (2) *Pheidole pallidula* uses a raid strategy like other species of this genus (Sjöstedt, 1907; Weber, 1964). The efficiency of attack is great because a soldier, fighting against an ant, remains with its predator for a long time, allowing the others to attack, in spite of the great toxicity of the frontal gland secretion. This species does not possess any venom in its poison gland.

The efficiency of geranyllinalool is about 100 times less than Fenthion, a commercial insecticide (Bayer Inc.) on *Schistocerca gregaria* (50 ppm) (Moreteau and Chaminade, 1983). If we consider the efficiency for 1 mg of insect, *Monomorium* sp., *Leptothorax parvulus*, and *Reticulitermes (lucifugus) banyulensis* show resistance (LD_{50} = 1/100 of their weight). In the contrast, geranyllinalool acts as a commercial insecticide for some species for which 1/200,000 of their weight is sufficient to achieve an LD_{50} in groups A and B. Competitive ants are less resistant than termite predators. However, termite feeding ants have evolved various detoxification abilities according to their attack strategy. Ants using raid and chemical crypsis are not able to detoxify this insecticide. In contrast, the major predator of termites (*Monomorium* sp. and *Leptothorax parvulus*) have very powerful detoxification mechanisms. This protection is average for nonspecific predators; their LD_{50} is generally in the range of the quantity emitted in a drop of termite soldier secretion.

Geographical Variation. In the Atlantic–Mediterranean area where *R. (l.)*

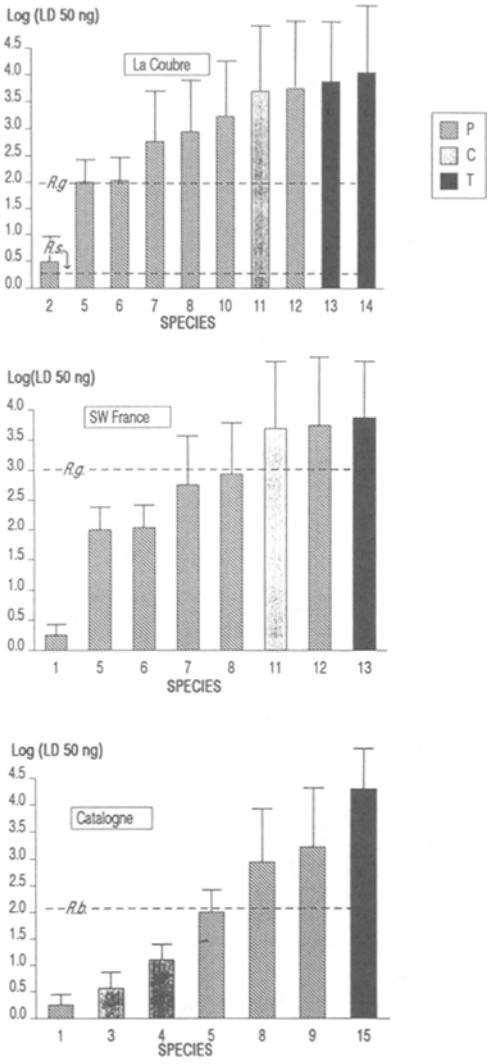


FIG. 2. LD_{50} values of geranyllinalool on the *Reticulitermes* termites and their ant predators and competitors LD_{50} values with confidence interval for $P = 0.95$. Broken line: average quantity of geranyllinalool emitted in the drop of a termite soldier. Hatching: predator P, Grey: competitors C, Black: termites T. La Coubre is in the first ecosystem where Rs and Rg1 are sympatric (LC, GV). SW France is the second Atlantic ecosystem where only Rg1 is present (AR, PY, BY). Catalogne is the Mediterranean ecosystem (BA) where R1b is allopatric with the two other species Ants—1: *Pheidole pallidula*, 2: *Hypoponera eduardi*, 3: *Leptothorax lichtensteni*, 4: *Leptothorax racovitzae*, 5: *Leptothorax unifasciatus*, 6: *Leptothorax nylanderi*, 7: *Myrmica rugulosa*, 8: *Aphaenogaster subterranea*, 9: *Crematogaster scutellaris*, 10: *Monomorium* sp., 11: *Lasius emarginatus*, 12: *Leptothorax parvulus*. Termites—13: *Reticulitermes (lucifugus) grassei*, 14: *Reticulitermes santonensis*, 15: *Reticulitermes (lucifugus) banyulensis*.

grassei (Rg2) is present, the number of predators is greater than in the other three ecosystems. On the other hand, whereas *R. santonensis* and *R. (l.) grassei* have to prevent attack and competition from many ant species, *R. (l.) banyulensis* contends with firmer and more sensitive ant species (*Pheidole pallidula*, *Leptothorax lichtensteni*, and *L. racovitzae*).

The geographical variation in the amount of geranyllinalool in the soldiers' secretion is coincident with the average resistance of the ants species. In the Atlantic area, the amount of geranyllinalool is high, and the ant predators and competitors are particularly resistant. The opposite situation is realized in the Mediterranean area, where a low level of diterpene alcohol is correlated with the lesser ant resistance (Figure 2).

We may consider soldier efficiency to be greater than the analysis of the LD_{50} has shown, because they use both mechanical and chemical weapons. Their mandibles generally retain ants protected by a strong cuticle and allow chemical weapons to reach their target, sometimes cutting a leg, petiol, or antenna. Their defensive function is limited to interspecific aggression, while workers play the major role in intraspecific aggression (Clément, 1978; Thorne, 1982).

This work showed a great diversity in predator and competitor resistance. The use of the same compound by wood and termites suggests a coevolutionary process of synthesis and detoxification that probably began in the first geological era. The molecular targets and detoxification mechanisms of geranyllinalool are now being investigated, and preliminary experiments indicate that it interferes with the neurohormonal system of insects.

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FURTHER STUDIES CONCERNING CHEMOATTRACTION AMONG FRY OF ARCTIC CHARR [*Salvelinus alpinus* (L.)] TO WATER CONDITIONED BY CONSPECIFICS

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Abstract—Chemoattraction of fry of Arctic charr [*Salvelinus alpinus* (L.)] to water scented by conspecifics of the same age was studied in a Y-maze fluviarium. The fry were offspring of two sympatric populations. Specimens of one of the two populations were indifferent to conspecific odor during the two-month experimental period. Specimens of the other population showed significant attraction to water scented by either population when their mean fresh weight was higher than 0.09 g. A significant difference in rheotactic response also was observed. Offspring of the population spawning in an inlet stream showed less motivation for upstream swimming, although they were significantly attracted to conspecific odor.

Key Words—Arctic charr, chemoattraction, conspecific odor, fry, rheotactic response, salmonids, *Salvelinus alpinus*, sympatric populations, Y maze.

INTRODUCTION

Previous studies have shown that chemoattraction to water conditioned by conspecifics is present in juveniles of landlocked Arctic charr [*Salvelinus alpinus* (L.)] (Höglund and Åstrand, 1973; Olsén, 1985a,b, 1986a, 1987, 1989; Olsén and Höglund, 1985) and in adults of anadromous charr (Selset and Döving, 1980). Three other species of salmonids did not induce attraction behavior in juvenile Arctic charr (Höglund et al., 1975), which supports the suggestion that the active substances are pheromones according to the definition of Karlson and Lüscher (1959). Furthermore, experiments have shown that ammonium and/

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or ammonia and amino acids that are emitted by juvenile Arctic charr are not acting as conspecific attactants (Olsén, 1986b,c). These substances instead might induce food search behaviour (Olsén et al., 1986).

In addition to Arctic charr, conspecific attractants have been demonstrated in various salmonid species, both among adult (Newcombe and Hartman, 1973; Emanuel and Dodson, 1979; Honda 1980, 1982) and juvenile specimens (Stabell, 1982, 1987; Quinn and Busack, 1985), but no study, except Olsén (1985b), deals with chemoattraction between fry. The aim of the present study was to examine when attraction to conspecific scent arises in Arctic charr. The charr were the offspring of wild specimens caught in two different spawning locations in Lake Torrön, Sweden and have been shown to be genetically separated (e.g., Hammar, 1980, 1984). The results of the present study indicate that the response of Arctic charr fry to conspecific odor and water currents is influenced by inherent components that reflect adaptation to the spawning area of the population.

METHODS AND MATERIALS

Experimental Fish. Hatchery-reared fry of Arctic charr, *Salvelinus alpinus* (L.), were studied. They were offspring of specimens caught in two spawning grounds in Lake Torrön, Jämland, Sweden. The spawning grounds are situated in the northern and southern parts of the lake, respectively, approximately 20 km apart (Olsén, 1986a). The charr from the southern locality (here called AB) spawn on reefs just outside the village of Åbränna. The adult specimens from the northern part were caught in the inlet stream called Holderströmmen (here called HO). The charr that spawn in the rivers Holderströmmen and Gånälven are considered to be distinct from the population spawning on reefs and shores in Lake Torrön (Hammar, 1980, 1984; Fürst et al., 1981; Hammar et al., 1983). The parents of the two groups tested might, accordingly, represent two different stocks (Ricker, 1972).

The embryos from the two populations were kept in separate troughs in a hatchery. Six hundred alevins each of HO and AB arrived at the Department of Zoophysiology in Uppsala on April 2, 1982, approximately one month after hatching. They were kept in dim light in two separate troughs with flowing aerated Uppsala tap water at the rate of 2 liters/min. Each stock was divided in three batches of 200 specimens each and placed in separate incubation boxes in their respective troughs. The light was turned on at 0700 hr and off at 1730 hr.

The fry started to attack food particles (Astra-Ewos F48 "char starter feed") approximately 16 days after arrival. They were fed twice a day (0800 hr and 1530 hr); the total amount was 3–4% of the body fresh weight per day. The lengths and weights of HO and AB specimens, respectively, at different occasions are shown in Table 1. The mortality of the two populations was very

TABLE 1. LENGTHS AND FRESH WEIGHTS OF SPECIMENS OF TWO POPULATIONS OF ARCTIC CHARR STUDIED

Date 1983	Population	
	Åbränna	Holderströmmen
March 3	Majority hatched	Majority hatched
April 2	Alevins arrived at the Department of Zoophysiology	Alevins arrived at the Department of Zoophysiology
April 18	Attacking food; some fry just below the water surface	Attacking food
April 21	ca. 50% swimming just below the water surface	ca. 5% swimming just below the water surface
April 23	0.07 ± 0.02 g, N = 11	0.06 ± 0.02 g, N = 10
April 28		Some excrement observed for the first time in compartment
April 30	0.07 ± 0.02 g, N = 10	0.07 ± 0.01 g, N = 10
May 2	Some excrement in compartment	
May 7	0.07 ± 0.03 g, N = 15; 26 ± 2 mm, N = 8; most fish two nares	0.07 ± 0.03 g, 13; 25 ± 2 mm, N = 13; most fish two nares
May 10	Induced mortality	Induced mortality
May 13	0.09 ± 0.04 g, N = 20	0.09 ± 0.04 g, N = 20
May 17		Some big specimens eating smaller
June 1	0.11 ± 0.06 g, N = 23; "hunger form" 0.03 g; some big eating small; nares differentiated	0.12 ± 0.08 g, N = 23 "hunger form" 0.03 g; nares differentiated
June 7	0.12 ± 0.09 g, 28 ± 3 mm, N = 10; silvery glitter on flanks	0.12 ± 0.06 g, 10; 28 ± 3 mm, N = 10; silvery glitter on flanks
June 24	0.23 ± 0.12 g, 33 ± 5 mm, N = 12	0.23 ± 0.11 g, 33 ± 4 mm, N = 11
July 15	0.38 ± 0.12 g, 38 ± 4 mm, N = 11	0.40 ± 0.18 g, 37 ± 5 mm, N = 11
July 30	0.59 ± 0.20 g, 43 ± 5 mm, N = 15	0.65 ± 0.28 g, 44 ± 6 mm, N = 15

low up to May 10 (cumulative mortality < 10 specimens), but increased and reached its maximum rate between May 11 and June 2 (3–24 specimens died per day in each population). The mortality was due to starvation as a lot of alevins did not start exogenous feeding. These "hunger forms" (Balon, 1980; Wallace and Aasjord, 1984a,b), which became thin as pins, were also attacked and ingested by the larger specimens, which grew much faster than others. Specimens from both stocks were studied later as 1-year-olds in an experiment concerning stock-specific odors (Olsén, 1986a).

Water Quality. All tests were performed in aerated Uppsala tap water, which is high in bicarbonate and calcium (Dimberg, 1988). The water temperature was equal in the two troughs and increased from 7.6 to 10.1°C during the experimental period, April 14–June 17, 1982. The oxygen concentration in the Y maze was 10.9 mg/liter (98% saturation).

Experiments Performed. "Up-swimming tests" were performed in dim light, 0.03 $\mu W/cm^2$, 0.01 lux, in a Y maze that has been described by Olsén (1985b, 1989). The flow of water in each inlet arm was 320 ml/min corresponding to 0.4 cm/sec.

Odor donors, 180–240 specimens, were placed in one of two compartments. The compartments supplied water (320 ml/min) to either of the two arms of the Y maze. The total amount of aerated water added to each compartment was 420 ml/min, the excess (100 ml/min) left by overflow through standpipes. The fry were fed 60 min before placement in the compartment. The first trial started 1 hr later. A single fry at a time was placed in the test area. After 5 min, the anterior (upstream) net was removed, and the fish was allowed to choose between pure tap water and conditioned water by swimming upstream. The test fish had made its choice when it swam up one arm, passing the trap therein. "Hunger forms," which were thin as pins, were not used as test fish, but they were not excluded as donors. Water conditioned by the presence of the odor donors was alternately distributed to either inlet arm of the Y maze. After testing each individual of a batch of five specimens, the supply of water from the two compartments was exchanged. The same procedure was followed in control tests with pure tap water from both compartments. Fry that did not move within 5 min after being placed in the test area were removed and the trial canceled. Trials were also canceled if the test fish did not move within 5 min after the upstream net had been removed or had not made a choice within another 10 min. Each day after the last trial, the two compartments and the Y maze were flushed with water and 95% ethanol. The total number of fry that preferred conditioned water or pure tap water were finally tested for deviation from a 1 : 1 ratio by a "heterogenity G-test" (Sokal and Rohlf, 1969). Differences in proportions were compared with another test according to Sokal and Rohlf (1969, "Testing equality of two percentages," pp. 607–608).

RESULTS

Growth and Mortality. No statistically significant differences in length and weight were observed between AB and HO during the period April 23–July 30, 1982 (Table 1). The cumulative mortality up to July 28 was 36% and 37% for AB and HO, respectively.

Behavioral Tests. Preference behavior of AB and HO are shown in Tables 2 and 3, respectively. The number of rejected fry are also given in these tables.

TABLE 2. PREFERENCE BEHAVIOR OF SPECIMENS IN ÅBRÄNNA POPULATION

No. and kind of odour donors[a]	Date 1983	No. of choices (%) for		No. of test fish rejected (% of total tested)
		Scented water	Pure water	
None (control)	April 14–15	16 (53)	14 (47)	3 (9)
180 AB	April 20–21	11 (37)	19 (63)	17 (36)
240 AB	April 26	15 (50)	15 (50)	2 (6)
240 HO	April 29–30	18 (45)	22 (55)	2 (5)
240 AB	May 2–3	16 (53)	14 (47)	2 (6)
240 HO	May 12–13	25 (63)	15 (37)	1 (2)
240 AB	May 17–18	20 (50)	20 (50)	4 (9)
240 AB	May 24	22 (55)	18 (45)	3 (7)
240 HO	May 25–26	19 (48)	21 (52)	4 (9)
240 AB	June 13–14	25 (63)	15 (37)	10 (20)
240 HO	June 16–17	18 (45)	22 (55)	15 (27)
Total AB		109 (52)	101 (48)	38 (15)
Total HO		80 (50)	80 (50)	22 (12)
Total AB + HO		189 (51)	181 (49)	60 (14)

[a]AB = Åbränna population; HO = Holderströmmen population.

TABLE 3. PREFERENCE BEHAVIOR OF SPECIMENS IN HOLDERSTRÖMMEN POPULATION

No. and kind of odor donors[a]	Date 1983	No. of choices (%) for		No. of test fish rejected (% of total tested)
		Scented water	Pure water	
None (control)	April 16 and 19	13 (43)	17 (57)	10 (25)
180 AB	April 21–22	11 (46)	13 (54)	7 (23)
180 HO	April 27	15 (60)	10 (40)	7 (22)
240 HO	April 28	23 (66)	12 (34)[b]	8 (19)
240 AB	May 3–4	16 (53)	14 (47)	8 (21)
240 HO	May 10–11	26 (65)	14 (53)[b]	25 (38)
240 AB	May 19 and 21	27 (68)	13 (32)[c]	19 (32)
240 HO	May 26–27	27 (68)	13 (32)[c]	6 (13)
Total HO		91 (65)	49 (35)[c]	46 (25)
Total AB		54 (57)	40 (43)	34 (27)
Total AB + HO		145 (62)	89 (38)[c]	80 (25)

[a]AB = Åbränna population; HO = Holderströmmen population.
[b]$0.05 < P < 0.1$.
[c]$P < 0.05$.

Number of Test Fish Rejected. A significantly higher proportion of the HO tested compared to AB were rejected, control tests included ($P < 0.001$; AB 63/463 = 0.136, HO: 90/354 = 0.254). The difference was high but not significant, however, if considering only the control tests, although the difference between the proportions was even greater ($0.05 < P < 0.1$; AB: 3/33 = 0.091. HO: 10/40 = 0.250).

Most fry were rejected because they did not move, but in the last three tests with AB as test fish and the last with HO, at least as many fry were rejected due to "no up-swimming choice within 10 min" as the number rejected due to lack of motion. The specimens rejected after 10 min had a high locomotor activity, swimming more freely up and down in both arms of the Y maze, and turning back when discovering the trap. They were less often in contact with the bottom and the walls.

Water Quality Preference. AB had no significant preference for water scented by either AB or HO, even when the total number of choices for conditioned and pure water are calculated (51% vs. 49%) (Table 2).

HO specimens were attracted to water conditioned by the presence of HO or AB in the last two tests ($P < 0.05$, Table 3). A high attraction ($0.05 < P < 0.1$) to scented water also was observed in two other tests with HO as donors (April 28, May 10 and 11). Significant attraction to water scented by charr was observed when testing the total number of choices made by HO in all tests ($P < 0.01$, Table 3). No significant difference was shown when comparing the proportion of HO preferring water conditioned by specimens of the same population (91/140 = 0.65) and AB (54/94 = 0.57), respectively.

A difference in preference behavior between HO and AB was shown when comparing the proportion of the total number of specimens that chose conditioned water (AB: 189/370 = 0.511, HO: 145/234 = 0.620; $P < 0.01$).

HO specimens were attracted with statistical significance to water conditioned by HO or AB when the mean fresh weight of both populations was between 0.09 and 0.12 g (Tables 1 and 3). HO specimens were attracted at a high rate ($0.05 < P < 0.1$) to water scented by their own population in tests performed on April 28 and May 10–11, respectively. The mean fresh weight of HO during these tests was between 0.07 and 0.09 g.

DISCUSSION

The present study demonstrates that chemoattraction to conspecific odor is present in free-swimming fry of Arctic charr [*Salvelinus alpinus* (L.)] with a fresh weight of 0.09–0.12 g (Holderströmmen, HO), but early fry of some populations (Åbränna, AB) may lack intraspecific attraction or they may be less motivated. In a previous study, strong attraction to conspecific odor was

observed in fry of a population from Lake Tinnsjön, Norway (Olsén, 1985b). The mean fresh weight of these fry was approximately 0.14 g, and they did not have, as in the above-mentioned specimens, a differentiated olfactory lamella (unpublished observations). Although fry of the AB population were not attracted to conspecific scent, they emitted attractants, as water scented by AB induced attraction in HO specimens.

Later on, as 1-year-olds, the specimens of the AB population were shown to have a significantly stronger attraction to water scented by their own population. They also were able to discriminate during simultaneous choice, chosing the water current scented by their own population, but the HO specimens were as strongly attracted to AB as to their own scent. Frequency analysis of serum esterases indicated that the HO specimens were not offspring of a discrete stock, which might explain their inability to discriminate (Olsén, 1986a).

The significance of the observed difference in chemoattraction behavior between the two populations of fry from Lake Torrön is not known. It is difficult to make any reliable hypothesis as the knowledge about the behavior and ecology of Arctic charr fry is scarce (Johnson, 1980). Chemoattraction might be of importance in social interactions during shoaling (Keenleyside, 1955; Hemmings, 1966; Höglund and Åstrand, 1973; Pitcher, 1979; Olsén, 1985a, 1989).

The observed difference in up-swimming motivation between HO and AB specimens might reflect a discrepancy in behavior of fry adapted to different spawning locations. HO specimens spawn in an inlet stream (Holderströmmen), and it is important for the fry to move to the rearing area that is located downstream (negative rheotaxis). Some studies have shown that fry of different salmonids might have an innate behavioral response to water currents (positive or negative rheotaxis), reflecting the location of the nursery or rearing area; i.e., if the spawning ground is located up- or downstream (e.g., Raleigh, 1971; Brannon, 1972; Kelso et al., 1981).

There might be a connection between attraction behavior and upstream movement, i.e., positive rheotaxis is induced by conspecific attractants (Stabell, 1984). Quinn et al. (1983) observed in a study dealing with intraspecific chemoattraction in coho salmon (*Oncorhynchus kisutch*) that adult specimens chose water conditioned by the presence of juveniles or conditioned by a supply of "home water" in preference for spring water. The adult coho were much more motivated to swim upstream in these tests than in control tests without attractive stimuli added to the spring water. But the proportion of fish making no choice at all was still at least 34% of the total number tested. It is reasonable to expect that adult specimens during the spawning migration are much more motivated for upstream movement when home stream odors are present. In the present study with fry of Arctic charr, no difference in motivation for upstream movement was observed between specimens tested in control tests with only tap water and those tested with conspecific odor, but there was a difference in moti-

vation between HO and AB. A significantly higher portion of the AB specimens, which did not discriminate between pure and scented water, was swimming upstream. In newly free-swimming fry, the location of the nursery area should be the most important factor in determining the behavior to the water current even though conspecific attractants are present. The observed differences in chemoattractive behavior and motivation for upstream movement between fry descending from charr caught in two different spawning localities support Hammar's conclusion, drawn from ecological data and gene frequency analysis of serum esterases (Hammar, 1984), that there are at least two sympatric populations in Lake Torrön, Sweden.

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VOLATILES FROM FECES OF WILD MALE HOUSE MICE
Chemistry and Effects on Behavior and Heart Rate

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Abstract—Volatile compounds from the fecal pellets deposited by a house mouse could be used for the purpose of communication with conspecifics. Analysis of volatiles from fresh and aged pellets by gas chromatography–mass spectrometry showed that many compounds were present and that ketones, alcohols, and carboxylic acids were the most common. Introducing volatiles from the fecal pellets of strange males into the territory of a singly housed male altered the site at which the resident mouse deposited most of its feces, and introducing the fecal pellets of a male mouse into the environment where it encountered a conspecific improved its success in an encounter with a stranger. The combined volatiles from feces of strange males had a greater effect on the heart rate of a male mouse than the combined volatiles from its own feces. Several individual volatiles from the fecal pellets of strange males affected the heart rates of test animals, but individual animals responded differently to constituents with similar retention times.

Key Words—Mouse, *Mus domesticus*, feces, fecal volatiles, volatile constituents, territoriality, heart rate.

INTRODUCTION

Fecal pellets deposited by all mammals are a rich source of volatile compounds. Fecal pellets of rabbits (*Oryctolagus cuniculus*), for example, contain more than

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100 volatile compounds (Goodrich et al., 1981a,b). Two lines of evidence suggest that, in this species at least, some of these compounds are of social significance: (1) Animals living in the wild deposit most of their feces on dunghills rather than depositing them haphazardly throughout their territories (Mykytowycz, 1968). (2) When two rabbits encountered one another in an environment containing the feces of one member of the pair, the donor rabbit made more exploratory forays and more nonaggressive contacts with its companion than the nondonor (Mykytowycz et al., 1976; Hesterman et al., 1981). The compounds responsible for the differences in the behavior of the donor and nondonor animals were not identified, but some of the volatile compounds from rabbit feces were shown to affect heart rate (Goodrich et al., 1981a).

In Australia, house mouse (*Mus domesticus*) populations occasionally irrupt into plagues, which cause serious economic losses and social and environmental problems (for reviews, see Redhead, 1988; Singleton and Redhead, 1989). Research on volatile compounds from the excretory products of house mice was instigated to determine whether attractants could be developed to facilitate control of, or possibly aid in the prevention of, plagues. The nature and social significance of volatiles from house mouse urine has been investigated extensively (for reviews, see Albone, 1984; Brown, 1985a,b), but the effects of volatiles from feces have been largely overlooked. In this paper, we report the results of chemical analyses carried out on the volatile components of both fresh and aged fecal pellets of male house mice. We also describe the effect of introducing the volatiles from the fecal pellets of strange males on the location at which singly housed males deposit their feces, the effect of introducing the fecal pellets from one mouse into an arena on the outcome of social encounters between the donor animal and its companion when both are introduced into the arena, and the effects of exposing males to the combined and the individual volatiles from the feces of strangers on their heart rates.

METHODS AND MATERIALS

Experiment 1—Chemical Composition

Under natural conditions, the volatile compounds from feces deposited by one individual could influence the behavior of another for some time after their deposition. Chemical analyses were therefore carried out on both freshly deposited feces and on feces kept at room temperature for 24 hr.

Methods. The fecal samples were obtained from wild male house mice trapped on the outskirts of Canberra. After capture, these animals were housed singly in laboratory cages, where they were supplied with commercial mouse food (Barastock) and water *ad libitum.* Air temperature in the animal room was 20 ± 2°C and day length was constant (reversed light–dark schedule, 12:12

hr). All mice spent at least one month in the room before being used as donors of fecal samples. By this time, they were at least two months old and their weights ranged from 18 to 25 g.

A fecal sample was collected as follows: three carefully washed cages were lined with filter paper, and a mouse was transferred to each cage. Each time an animal defecated during the 10-min period following transfer, the pellet was removed and placed in a clean container. This pooled sample was then divided into two; the first subsample was processed immediately after collection (fresh sample), the second was allowed to remain at room temperature (25°C) for 24 hr before being processed (aged sample).

The technique used to collect the volatiles from the fresh and aged fecal samples on Chromosorb 105 (Johns-Manville) for examination by gas chromatography (GC) and gas chromatography–mass spectrometry (GC-MS) was similar to that described previously by Goodrich et al. (1978, 1981a). Oxygen-free nitrogen (40 ml/min) was passed over the sample (0.5 g), which stood in a 40°C water bath, for 30 min. The effluent was collected in a single preconditioned trap containing a bed of Chromsorb 105 (50/60 mesh, 100 mg) packed into a stainless-steel tube (90 mm, 3.2 mm OD). The tube was then purged with dry nitrogen (40 ml/min for 15 min) to remove residual water, sealed with Teflon end-caps, and stored over solid carbon dioxide until required.

Gas chromatography was conducted on a Varian 3400 instrument equipped with a flame ionization detector with modifications similar to those described previously (Murray, 1977). The headspace volatiles were introduced at 170°C over a period of 10 min. For qualitative comparisons between samples, the GC conditions were as follows: bonded phase (BP 20), vitreous silica capillary column (25 m, 0.33 mm ID), 1 ml H/min, 50°C/8 min, to 160°C at 10°C/min; *n*-undecane was added to sample as internal standard.

The volatile components of the sample were identified through capillary GC-MS (Kratos MS 25 double focussing mass spectrometer), using electron impact ionization at 70 eV. The GC conditions were identical to those described previously. Identifications were confirmed, where possible by comparison with mass spectra and linear retention indices (LRI) of pure samples.

Results. Figure 1 shows the gas chromatograms of the volatiles collected from fresh and aged feces, and Table 1 lists the compounds that were identified. The volatiles from the fresh sample included an aldehyde, ketones, alcohols, carboxylic acids, and phenol. Many of the compounds found in the fresh sample were also found in the aged sample, but the volatile fatty acids were present in smaller concentrations in the former than in the latter. The aged sample also included compounds that were not detected in the fresh sample (see Table 1).

Discussion. These results shown that the fecal pellets of house mice, like those of rabbits (Goodrich et al., 1978, 1981a), are a rich source of volatile compounds. They also show that the compounds released from feces may change

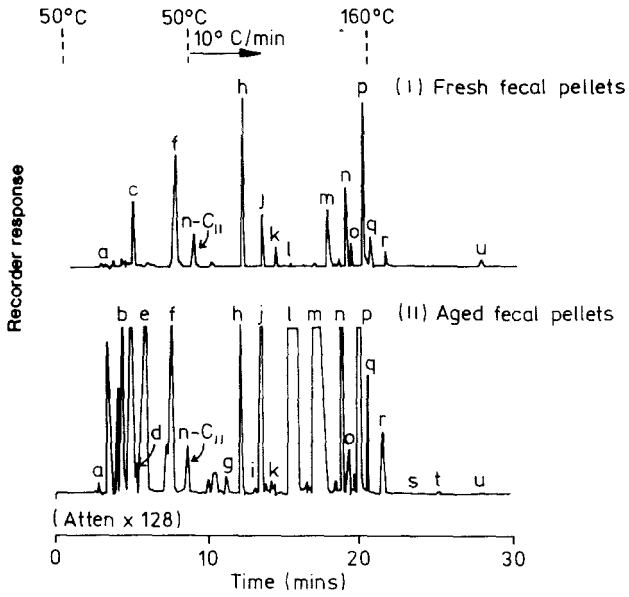


FIG. 1. Gas chromatographic comparison of headspace volatile components collected from freshly voided fecal pellets of wild male house mice and from feces of the same animals kept at 25°C for 24 hr. Letters refer to compound identifications in Table 1.

with time, presumably as a result of the activity of the gut microbial flora excreted with the food remains.

Experiment 2—Fecal Volatiles and Fecal Deposition

In the rabbit, the presence of volatiles from the feces of one or more individuals appears to encourage others to defecate at the same site, for each territory usually includes several dunghills where territory members deposit most of their feces (Mykytowycz, 1968). In this experiment we examined the effect of volatiles from the feces of strange conspecifics on the distribution of fecal pellets in territories occupied by singly housed male mice.

Methods. The 12 wild males used as test animals were handled in the same way as those used in experiment 1. Fecal samples to be used as a source of volatiles were collected by placing each of the test mice in a cage with a wire mesh floor for 16 hr. Uncontaminated pellets deposited on the blotting paper beneath the cages of the 11 animals not being used in the test were pooled and stored at −18°C until needed.

The apparatus used to monitor the pattern of deposition of fecal pellets was an arena (30 × 30 × 30 cm) with Plexiglas walls and a floor of wire mesh over

TABLE 1. VOLATILE COMPOUNDS IN HEADSPACE GASES FROM FRESH AND AGED FECAL PELLETS OF WILD MALE HOUSE MICE[a]

	Compound	Type of fecal pellet[b]	
		Fresh	Aged
a	Ethanal	+	+
b	Butan-2-one	−	+
c	Ethanol	+	−
d	Ethyl propanoate	−	+
e	Butan-2,3-dione	+	+
f	n-Propanol	+	+
g	3-Methylbutyl ethanoate	−	+
h	*n*-Butanol	+	+
i	3-Methylbutyl propanoate	−	+
j	3-Methylbutan-1-ol	+	+
k	*n*-Pentanol	+	+
l	3-Hydroxybutan-2-one	+	+
m	Ethanoic acid	+	+
n	Propanoic acid	+	+
o	2-Methylpropanoic acid	+	+
p	*n*-Butanoic acid	+	+
q	3-Methylbutanoic acid	+	+
r	*n*-Pentanoic acid	+	+
s	*n*-Hexanoic acid	−	+
t	2-Phenylethanol	−	+
u	Phenol	+	+

[a] The gas from the fresh sample was collected immediately after the pellets were deposited, the gas from the aged sample was collected after the pellets had been kept at 25°C for 24 hr.
[b] + indicates that the compound was detected, − that it was not detected.

a sheet of blotting paper. The blotting paper was divided into three sections (each 30 × 10 cm) designated areas 1, 2, and 3. A shelter equipped with food and water was located over the center of area 2.

The fecal deposition pattern of each test mouse was measured twice: once when the only fecal volatiles present were those of the test mouse itself and once when a sample of feces from a group of males was positioned along one side of the arena. At 1630 hr on day 1, the test mouse was introduced into the arena and an airstream (200 ml/min) was directed into the arena at floor level from the long side of area 3. The mouse was allowed to remain in the apparatus in total darkness until 0830 hr the following day, when the arena was lifted from above the blotting paper and the numbers of fecal pellets in areas 1, 2, and 3 were counted. At 1630 hr on day 2, the apparatus was reassembled with fresh blotting paper beneath the arena, the test mouse was reintroduced, a tube

of stainless-steel mesh containing 2 g of male feces was placed along the wall adjacent to area 3, and an airstream was directed into the arena over the fecal sample. At 0830 the following morning fecal pellet numbers in areas 1, 2, and 3 were counted.

The significance of the difference between the numbers of pellets deposited in each of the three areas on day 1 and the numbers deposited on day 2 was determined by applying chi-square tests to the data for each of the 12 test mice.

Results. When the only volatiles present were those from the test mice themselves, the test animals deposited over 75% of their fecal pellets in and around the shelter, i.e., in area 2 (Table 2). The remaining pellets were deposited in about equal numbers in areas 1 and 3. When the feces of strange males were present, all mice deposited more feces in area 3 than in areas 1 and 2. In all instances the differences between the numbers deposited in the three areas on days 1 and 2 were significant; all chi-square values were greater than 11.0 ($P < 0.001$).

Discussion. These results show that the distribution of fecal pellets within the territories of singly housed male house mice can be affected by introducing volatiles from the fecal pellets of strangers into their territories.

Experiment 3—Fecal Volatiles and Outcome of Social Encounters

Mykytowycz and coworkers showed that, when a rabbit was confronted with a stranger in an environment containing the feces of one member of the pair, the donor animal usually made more exploratory forays than the nondonor and initiated contact with its companion more frequently (Mykytowycz et al., 1976). The effect of the presence of fecal pellets from one mouse on the outcome of social encounters between pairs of house mice was examined by carrying out experiments similar to those carried out on rabbits.

TABLE 2. MEAN NUMBER (± SE) OF FECAL PELLETS DEPOSITED IN TEST ARENA BY WILD MALE HOUSE MICE (12) OVER A 16-HOUR PERIOD ON DAYS 1 AND 2, AND MEAN PERCENTAGES DEPOSITED IN EACH OF THREE AREAS BENEATH ARENA[a]

	Mean number	Mean percentage		
		Area 1	Area 2	Area 3
Day 1	112 ± 9.0	14 ± 9.5	77 ± 3.6	10 ± 3.2
Day 2	122 ± 15.4	8 ± 1.4	17 ± 2.5	75 ± 3.3

[a] On day 1 clean air flowed into the arena along the outer margin of area 3; on day 2 the air flowing into the arena passed over fecal pellets (2 g) collected from males that were strangers to the test animal.

Methods. The 32 wild mice used as test animals in this experiment were handled in the same way as those used in experiment 1. All were adults and all were housed singly for at least one month before thay were tested. A week before the first test was carried out the animals were divided into two subgroups (groups 1 and 2), which were housed in separate rooms. For each mouse of a given weight in group 1 there was a mouse of similar weight in group 2. Fecal samples, which were as free of urine as possible, were collected from each mouse by housing the animal in a cage with a wire mesh floor kept overnight over a clean sheet of blotting paper. The pellets were collected the following morning, transferred to vials and stored at −18°C.

The circular arena (50 cm diameter) used in this experiment (Figure 2) was similar to the one use in experiments carried out with rabbits (Hesterman et al., 1981; Goodrich et al., 1981a). Before each test, the floor and wall of the arena were lined with clean polythene sheeting (0.1 mm thick). The upper end of the polythene sleeve, which lined the Plexiglas cylinder forming the wall, was attached to the inlet head, which contained the two antechambers for holding the mice prior to their introduction into the arena. Each of these antechambers had one door at the top for introducing the test animal and a second door below

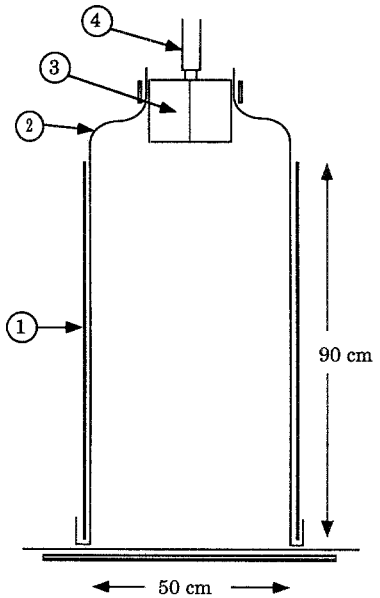


FIG. 2. Arena used to observe the behavior of a wild male house mouse confronted with a strange conspecific in the presence of its own feces or the feces of its companion. 1, Plexiglas cylinder; 2, polythene sleeve; 3, inlet head with two antechambers for holding the mice prior to their introduction into the arena; 4, air inlet pipe.

for introducing the mouse into the arena. This second door could be operated by remote control. The inlet head was kept in position by suspending it from three wires attached to a stand and positive pressure was maintained in the chamber throughout each test by introducing an air stream through a pipe in the inlet head.

The effects of fecal volatiles on the outcome of social encounters between house mice was measured by introducing the feces of one animal into the test arena and comparing the behavior of that animal (the donor) with the behavior of a second animal (the nondonor) when both were placed in the arena. At the start of each test, a fecal sample (0.5 g) from one of the mice from Group 1 was introduced into the arena. The inlet head was then placed in position, and the donor mouse (with a strip of reflective tape on its tail) and the nondonor mouse (the mouse from group 2 whose weight was similar to that of the donor) were introduced into the two antechambers. When all was in readiness, the room was illuminated with infrared light. After the mice had been allowed to adjust to their new surroundings for 15 min, both were introduced into the arena by opening the doors beneath the antechambers. The subsequent behavior of both mice was viewed, from an adjoining room, by two observers using a video camera fitted with an image intensifier (Philips). Both observers recorded the incidence and duration of three types of activity: exploratory forays (locomotion about the test chamber), nonagressive contacts (sniffing, grooming, nudging), and aggressive contacts (fighting, chasing). [The definitions of these activities were those adopted by Oortmerssen (1971) and Mykytowycz and Dudzinski (1972).] Observations were made over a 10-min period starting from the first encounter between the two animals. About two weeks later, the test was repeated with the same pair of mice but with the feces of the second mouse in the chamber.

In this experiment, all mice were tested twice: once in the presence of their own feces (donor situation) and once in the presence of the feces of their test-partner (nondonor situation). For each of the behavioral activities recorded, means were calculated for the donor and nondonor situation and means and variances for the donor–nondonor differences. To minimize the effects of between-mouse differences in activity, the variances were calculated for the sums of the donor–nondonor differences for each pair of mice. Single-sample t tests were used to determine the significance of the donor–nondonor difference for each activity. The tests were carried out on the tranformed data using the formula:

$$\text{Log}_e[(\text{donor score} + 1)/(\text{nondonor score} + 1)]$$

Chi-square tests were used to test the significance of the effects of the presence of their own or their test-partner's feces on the number of mice initiating aggressive interactions.

Results. Neither the mean number nor the mean duration of the exploratory forays made by the donor and the nondonor mice appeared to be affected by the source of the fecal sample placed in the test chamber at the start of the test (Table 3). The mean number and mean duration of the nonaggressive contacts made by the donors and nondonors, too, appeared to be unaffected by the source of the sample, but both the mean number and the mean duration of the aggressive contacts made by the donor animals were significantly greater than those made by the nondonors. However, the difference in the numbers of donors and nondonors *initiating* aggressive attacks was not significant (donors, 31/32, nondonors 27/32; chi square = 2.94, NS).

Discussion. These results suggest that house mice, like rabbits, are more likely to succeed in social encounters with conspecifics in environments where the fecal pellets are their own than in environments where the fecal pellets are from strangers.

Experiment 4—Fecal Volatiles and Heart Rate

In rabbits, some of the volatiles from fecal pellets were found to affect heart rate when presented to test animals (Goodrich et al., 1981a). In this experiment we examined the effects of fecal volatiles on the heart rate of the house mouse. In one test, the mice were exposed to the combined volatiles from their own feces and to the combined volatiles from the feces of strangers. In the second test, the mice were exposed to the chromatographically fractionated components of the headspace from a pooled sample of fecal pellets.

Methods. Four wild males were used to determine the effects of combined volatiles from their own and strange male feces on heart rates, and seven wild males were used to determine the effects of the fractionated components of the volatiles from strange male feces on heart rates. The mice were handled in the same way as those used in experiment 1. The method used to obtain fecal samples from individual mice was the same as that described in experiment 3; the method used to obtain pooled samples from several mice was the same as that described in experiment 2.

The heart rates of the test mice were measured by recording their ECGs. Pin electrodes were attached to the skin over the sternum and the skin over the shoulders. The electrical signals, which were transmitted via a miniature radio transmitter to a high-quality FM radio/casette recorder, were processed through a biotachometer (Narco Biosystems Inc. BT/1200), which produced an output voltage proportional to the beat-to-beat heart rates. These rates were recorded on a multipen strip-chart recorder running at 140 mm/min.

The heart rate response of the test mice to the fecal volatiles was measured by a technique similar to that used for rabbits (Hesterman et al., 1976). The test mouse was lightly anesthetized with ether and the electrodes attached. The

TABLE 3. MEAN NUMBER AND MEAN DURATION (SEC) OF EXPLORATORY FORAYS, NONAGGRESSIVE CONTACTS, AND AGRESSIVE CONTACTS BY WILD MALE HOUSE MICE OVER 10-MIN PERIOD WHEN TWO MICE WERE PLACED IN TEST CHAMBER CONTAINING FECAL PELLETS OF ONE OF THEM (DONOR), TOGETHER WITH MEAN DIFFERENCES (± SD) BETWEEN SCORES WHEN TEST MOUSE WAS DONOR AND SCORES WHEN IT WAS NONDONOR

	Donor situation		Nondonor situation		Mean difference (± SD) (donor − nondonor)	Significance of difference	
	Occurrence	Mean score	Occurrence	Mean score		t	P
Exploratory forays							
Incidence	32	45.0	32	42.8	2.2 ± 41.2	0.90	NS
Duration		130.9		130.8	0.1 ± 97.3	−0.22	NS
Nonaggressive contacts							
Incidence	31	11.0	29	13.8	−2.7 ± 13.6	−1.46	NS
Duration		28.7		31.0	−2.3 ± 36.2	0.50	NS
Aggressive contacts							
Incidence	31	31.5	27	18.2	13.3 ± 42.0	3.22	<0.01
Duration		45.4		25.7	19.7 ± 57.0	2.99	<0.01

animal was then placed in a comfortable position in a restrainer (a mold in the shape of a mouse in the crouched position), the mask attached to the gas line was placed over its nose, and the animal and its attachments were transferred to a soundproof box (30 × 25 × 25 cm) equipped with an exhaust fan, which removed unwanted volatiles, including the ether used to anaesthetize the animal. The test animals quickly regained consciousness but the tests were not begun until after the animals had been in the apparatus for at least 15 min.

Mice were exposed to the combined volatiles from their own feces and the feces of strangers by passing compressed air (150 ml/min) over a fecal sample (2.0 g) that was kept in a flask maintained at 36°C. The volatiles from each sample were presented for 1 min after a 10-min control period when the air flowed over distilled water. The mice exposed to the fractionated volatiles from the feces of strangers were exposed to clean air for 10 min before being presented with successive fractions from the fecal sample. These fractions were prepared by collecting the headspace gas from the sample, introducing this onto the chromatographic column (glass SCOT column coated with Carbowax 20 M (60 m, 0.5 mm ID), splitting the effluent 5:1, and directing the minor portion of the flame ionization detector and the remainder to the test mouse. The output from the GC detector and the output from the biotachometer were displayed on the same chart recorder.

Both the combined volatiles and the fractionated volatiles that affected heart rate caused deceleration. The changes induced were considered significant if the percentage decrease in rate was 15% or greater (Goodrich et al., 1981a).

Results. Both exposure to the combined volatiles from their own fecal pellets and exposure to the volatiles from the fecal pellets of strange males caused a slowing of the heart rate in the four test mice, but the depression in heart rate caused by volatiles from the pellets of strange males (mean, 43%; range 39–48%) was greater than that caused by volatiles from their own feces (mean, 13%; range 0–24%). An example of the response of one mouse to the volatiles from the two sources is shown (Figure 3).

When the mice were exposed to clean air, heart rate was slowed in only one of the seven mice tested, and the rate returned to normal within a few seconds. When the mice were exposed to the effluent of the GC column, the heart rate of each mouse was only reduced by 15% or more by a few of the fractions (Figure 4). The fractions affecting heart rate appeared to vary from mouse to mouse, for fractions with the same retention times affected some animals but not others. Fractions with retention times similar to those of the volatile fatty acids appeared to depress heart rate for longer than those with shorter or longer retention times.

Discussion. The results of the experiment where the test mice were exposed to the combined volatiles from fecal pellets suggest that heart rates in this species are affected more by volatiles from the feces of strangers than by volatiles

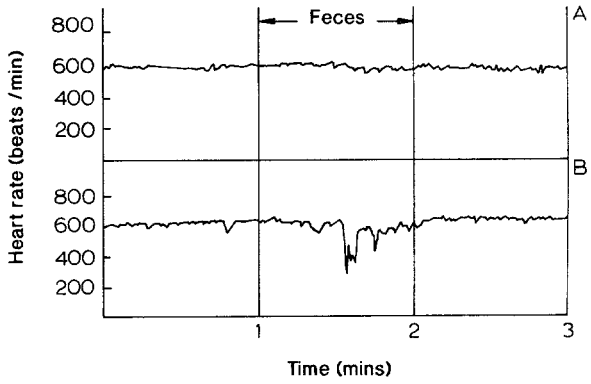


FIG. 3. The effect of volatiles from its own fecal pellets and of volatiles from the fecal pellets of a stranger on the heart rate of a wild male house mouse.

from their own feces. The results of the experiment where the test mice were exposed to the fractionated volatiles from the feces of strangers suggest that, in most mice, heart rates are only affected by a few of the volatiles given off from feces and that volatiles that affect the heart rate of one individual may not necessarily affect the rate in another.

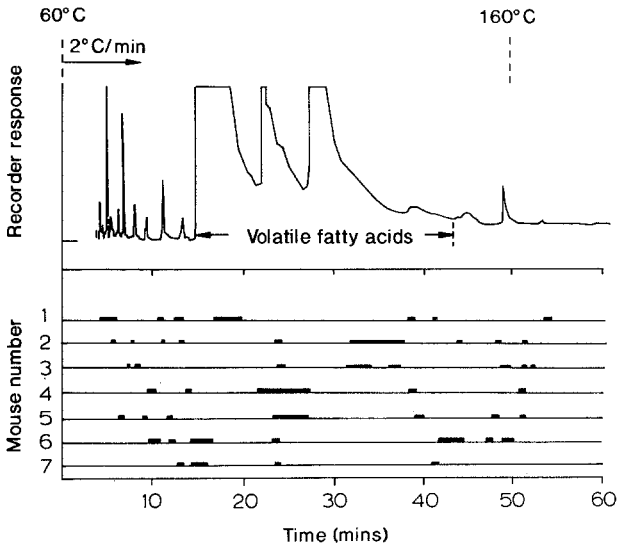


FIG. 4. Gas chromatogram on Carbowax 20 M glass SCOT column of headspace volatiles from a pooled sample of fecal pellets from wild male house mice together with indications of the periods at which heart rate was depressed (by 15% or more) in each of the seven test mice.

RESULTS AND DISCUSSION

The results of experiment 1 show that fecal pellets of house mice, like those of rabbits, are a rich source of volatile compounds, and the results of experiments 2, 3, and 4 suggest that in house mice, like rabbits, these volatile may affect the site at which an individual defecates, its success in social encounters, and its heart rate.

Many of the volatiles found in the headspace gas from fresh and from aged feces of house mice were also found in the headspace gas of rabbit feces (Goodrich et al., 1981a,b), but others were detected only in gas collected from mouse feces. Some of the differences between the fecal volatiles of the two species may have been due to differences in diet and to differences in the nature of the glands discharging at or near the anus. Commercial mouse food contains less green matter and more grain than commercial rabbit food and the anal glands of mice are sebaceous (Hummel et al., 1966), whereas those of rabbits are serous (Mykytowycz, 1968). Differences in the microbial flora found in the gut and in the skin glands of the two species also may have contributed to the between-species differences because many of the compounds found in the gases collected from feces and from the secretions of exocrine glands are a consequence of microbial activity (Albone, 1984).

Observations on undisturbed populations of rabbits and house mice suggest that volatiles from feces may have a greater effect on the choice of defecation sites in the former than in the latter. Most rabbits usually defecate on the dunghills scattered about the territory (Mykytowycz, 1968), but in house mice defecation occurs most commonly at locations where the animals spend most of their time, namely, in and around shelters and feeding sites and along routes followed during exploratory forays (near walls and other upright objects and along structures bridging gaps separating one part of a territory from another) (Welch, 1953; Pennycuik, 1973; Hurst, 1987). The effect of volatiles from the feces of strange males on the pattern of fecal deposition in the territories or our singly housed males, therefore, may well have been due to the effect of the volatiles on the time spent in different parts of the territory rather than to a deliberate attempt on the part of the mice to deposit their feces on or near those of other males. Hurst's (1987) observation that the residents of a territory failed to overmark the deposits of strangers left on objects placed in their territories would support this view.

Volatile compounds from material deposited by strangers have been observed to affect the way in which both rabbits and house mice behave in the presence of conspecifics from different social groups (Mykytowycz, 1968; Harrington, 1976), and Mykytowycz and coworkers have shown that volatiles from fecal pellets can affect the outcome of social encounters between pairs of rabbits placed in an otherwise neutral arena (Mykytowycz et al., 1976; Hesterman et

al., 1981). Our results show that in house mice, too, fecal volatiles may affect the outcome of social encounters between males: mice tested in the presence of their own feces attacked their companions more frequently and for longer than mice tested in the presence of feces from a stranger. Attack latencies in house mice are known to be affected by the prior social experience of the animals and by their age (Zegeren, 1980), but these factors were of little importance in the present experiment because all mice were housed singly for several weeks before the first test was carried out, and only two weeks elapsed between the first and second tests.

When rabbits were exposed to the combined volatiles from the feces of strangers, their heart rates usually declined (Hesterman et al., 1976). Exposing mice to the combined volatiles from feces also caused a decrease in heart rate, but volatiles from the feces of strangers were more effective than volatiles from the feces of the test mice themselves. This difference in the responses of the test mice to their own and foreign volatiles must have been due to between-mouse differences in the composition of the secretions from glands associated with the digestive tract, for all mice were fed on commercial mouse food for several weeks before the fecal samples were collected. The ability of the test mice to react to between-mouse differences in the composition of the combined volatiles from their own and foreign feces could have been due to quantitative or qualitative differences between the volatiles from the two sources because these differences could affect the responses of the test mice directly or via their effects on synergism between a number of stimulus molecules.

When rabbits were exposed to the fractionated volatiles from the feces of strangers, their heart rates were slowed by some and accelerated by others, but fractions that affected the heart rate in one individual often had little effect on the heart rates of others (Goodrich et al., 1978, 1981a). In house mice, some fractions appeared to cause cardiac deceleration, but none were associated with an increase in heart rate and in mice, like rabits, compounds that affected heart rate in one individual frequently failed to affect heart rate in others. These between-mouse differences may have been due to individual differences in sensitivity to a given compound, as strain differences in sensitivity are known to exist (Price, 1977; Wysocki et al., 1977). Differences in the olfactory environments or the social experiences of the mice prior to capture also may have contributed to between-mouse differences, because exposure to a compound early in life in association with a pleasant or unpleasant experience is known to affect the response of test animals to the same compound alone in later life (see Doty, 1986, for review). The causes of between-mouse differences in heart rate and the significance of these differences clearly need further investigation.

The results of experiments 2–4 strongly suggest that house mice may use volatiles from feces as one of their sources of information about their social environment. Fecal volatiles, therefore, could be a potential source of com-

pounds that could be used to control mouse numbers. The addition of feces to a diet does not appear to affect its palatability (Pennycuik and Cowan, 1990), but the effects of incorporating feces, or the volatiles from feces, into products designed to attract or repel mice have yet to be investigated.

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VOLATILE COMPOUNDS FROM EXCRETA OF LABORATORY MICE (*Mus musculus*) Preliminary Examination of Composition and Effects on Behavior

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Abstract—Volatile components of all the excretory products deposited by house mice may contribute to their ability to identify their own territories. When mice were placed on a clean surface, they deposited feces, secretions discharged from the anus (anal secretion) and urine. Exposure to several clean surfaces in succession caused a decline in the number of fecal pellets and urine spots deposited and an increase in the number of anal smears. The volatile compounds emanating from feces and anal secretion appeared to be qualitatively and quantitatively different from those emanating from urine, but many compounds with short retention times appeared to be common to feces and anal secretion. Introducing volatiles from feces of strange males into the territory of a singly housed male altered the site at which the resident animal deposited its feces but had no effect on the site at which it urinated. Introducing the feces or the anal secretion of a male mouse into the environment where it encountered a strange conspecific appeared to improve its success in encounters with a conspecific. It was concluded that at least some of the volatile compounds that enable mice to distinguish their own territory from those of neighboring groups may be derived from feces and that many fecal volatiles may originate from the secretions of glands opening into the digestive tract.

Key Words—Mouse, *Mus musculus*, feces, urine, volatile constituents, gas chromatography, behavior.

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INTRODUCTION

House mice (*Mus musculus*) living in pens or as commensals in farm buildings establish territories that are defended by the resident mice (e.g., Crowcroft, 1955; Crowcroft and Rowe, 1963; Reimer and Petras, 1967; Singleton and Hay, 1983). Recognition of the boundaries of adjacent territories is thought to be due, in part at least, to the presence of volatile compounds emanating from the excretory products of the resident animals, for (1) dominant males are reluctant to enter areas soiled by a neighboring dominant (Mackintosh, 1973; Harrington, 1976), (2) both males and females prefer volatiles from their own demes to volatiles from neighboring demes (Cox, 1984), and (3) juveniles approach objects marked by mice from neighboring territories with extreme caution (Hurst 1987). Indirect evidence suggest that urine may be one of the sources of the volatiles responsible for territory recognition; dominant males deposit urine throughout their living space (e.g., Desjardins et al., 1973), and these deposits reduce the amount of time spent in the vicinity by subordinate males (Jones and Nowell, 1973a,b; 1977). Furthermore, subordinate mice (females or castrate males) marked with the urine of a dominant male are attacked more frequently by intact males than unmarked controls (Mackintosh and Grant, 1966; Mugford and Nowell, 1970).

In undisturbed habitats, house mice deposit both urine and feces in and near shelters and feeding sites and along routes followed during exploratory forays (Welch, 1953; Pennycuik, 1973: Hurst, 1987). Experiments with wild mice suggest that fecal volatiles, like volatiles from urine, may be involved in the demarcation of territorial boundaries, because the presence of fecal volatiles from strangers appears to affect their behavior (Goodrich et al, 1990). In the experiments reported here, we identified the materials deposited from the anus (feces and secretions from glands associated with the digestive tract–anal secretion) by laboratory mice (BALB/c), separated and compared the volatiles from these materials and from urine, and examined the effects of the presence of feces and anal secretion on the sites chosen by mice for urination and defecation and on the outcome of encounters between conspecifics.

METHODS AND MATERIALS

Experiment 1—Response to Clean Substratum

House mice transferred to a clean arena quickly soil their surroundings. In this experiment we identified the excreta eliminated by the experimental animals following transfer to clean surroundings and measured the temporal changes in the amounts deposited.

Methods. BALB/c (An Bradley/Wehi) males, obtained from the Australian

National University, Canberra, were used in this and subsequent experiments. All animals were adults and weighed 28–35 g. For at least four weeks prior to each experiment, the animals were housed singly in laboratory cages where they were supplied with commercial mouse food (Barastock) and water ad libitum. Air temperature in the animal room was 20 ± 2°C and day length was constant (reversed light–dark schedule, 12 : 12 hr).

Excreta deposited by mice transferred to clean surroundings were identified by placing each test animal in an arena covered with clean filter paper for 10–15 min, noting the nature and location of the materials deposited during this period and observing the marks left by these materials on the filter paper after the mouse had been removed from the arena.

Temporal changes in the quantities of urine, feces, and anal secretion deposited by the test animals were measured by moving the mice to five clean areas in succession. These measurements were made in an arena (15 × 160 cm) lined with clean paper and subdivided into five equal sections (15 × 40 cm) with four movable glass partitions (15 × 15 cm). The arena was housed in a room lit with a dim red light. A video camera, fitted with an image intensifier (Philips) enabled an observer in an adjoining room to view the arena and its occupant. Following transfer to the arena, the mouse spent 10 min in each of the five sections. Transfer from one section to the next was effected by removing the partition separating the two, allowing the mouse to move to the next section and replacing the partition. During the course of the test, the observer recorded the number of times the animal dragged its anus on the substratum in each section and the time spent on this activity and, after the mouse had been returned to its cage, the number of excretory products of each type present on the surface of the paper lining each section.

Results. The test mice explored their new surroundings as soon as they were transferred to the clean surface. During these forays, they were observed to urinate and to defecate and to rub clear fluid from the anus onto the substratum (Figure 1). Subsequent examination of the surface showed that the animals had left faintly colored spots with an even, roughly circular outline where they had urinated and shiny, yellowish, elongated smears where they had deposited fluid from the anus.

When mice were transferred to the first clean area of the test arena, they deposited appreciable quantities of urine, feces, and anal secretion throughout the area during a 10-min period (Table 1). All three excretory products were also deposited in the remaining areas but the numbers of urine spots and fecal pellets in areas 2–5 were significantly smaller and the numbers of anal smears significantly greater than those in area 1. This increase in the number of smears was accompanied by an increase in the number of times the mice dragged the anal area on the substratum and an increase in the amount of time spent on this activity.

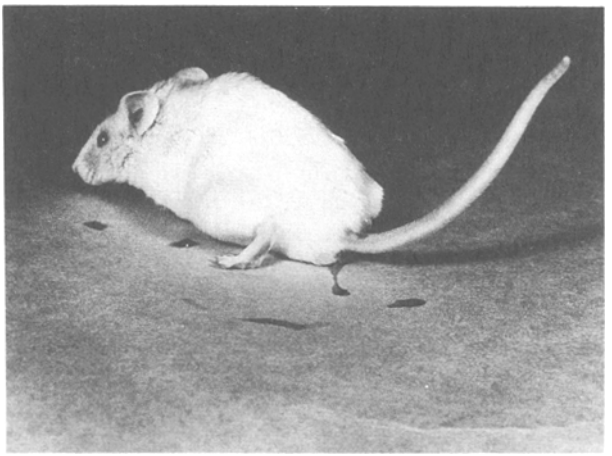


FIG. 1. House mouse (BALB/c male) depositing secretions from glands associated with the digestive tract (anal secretion) onto the clean paper lining the arena.

Discussion. These results show that house mice deposit anal secretion as well as urine and feces within their surroundings. They also suggest that, when mice are confronted with objects free of volatiles from excretory products, they tend to deposit excreta on the object deliberately and that the majority of these deposits are materials discharged from the anus.

Experiment 2–Chemical Composition of Urine, Feces, and Anal Secretion

Headspace gases from both the urine and feces of house mice are known to be rich in volatile compounds (Schwende et al., 1986; Goodrich et al., 1990). In this experiment the compounds in the headspace of feces were compared with those in the headspaces of anal secretion and urine. Some of the volatile compounds from feces were identified and anal secretion was tested for the presence of protein and carbohydrate.

Methods. Samples of urine, feces, and anal secretion were obtained from three adult BALB/c males. Fecal samples were collected by placing the mice in clean cages lined with filter paper and removing the pellets with forceps as soon as they were deposited. Anal secretion was obtained by gently massaging the anal region of the mouse with a glass rod, collecting the liquid appearing at the anal opening in a glass capillary tube, and transferring this to a vial where it was diluted with an equal quantity of distilled water to prevent it from forming an insoluble translucent mass. Urine was collected by gently squeezing the bladder of the mouse and collecting the sample expelled in a clean glass container. All samples were either analyzed immediately after collection or stored at $-18°C$ until required.

TABLE 1. MEAN NUMBER (±SE) OF URINE SPOTS, FECAL PELLETS, AND ANAL SECRETION SMEARS DEPOSITED BY MALE BALB/c MICE (20) OVER FIVE SUCCESSIVE 10-MINUTE PERIODS IN FIVE IDENTICAL CLEAN AREAS, TOGETHER WITH MEAN NUMBER OF OCCASIONS ON WHICH THEY WIPED THE ANAL AREA ON SUBSTRATUM AND MEAN TIME SPENT ON THIS ACTIVITY

	Area 1	Area 2	Area 3	Area 4	Area 5	Significance of difference	
						f	*P*
Number of							
Urine spots	2.5 ± 0.65	0.4 ± 0.11	0.6 ± 0.11	0.4 ± 0.11	0.5 ± 0.18	8.05	<0.001
Fecal pellets	8.7 ± 0.68	2.8 ± 0.20	2.3 ± 0.36	2.5 ± 0.33	2.2 ± 0.31	56.35	<0.001
Smears	4.0 ± 0.58	6.7 ± 0.55	8.2 ± 0.62	8.3 ± 0.90	10.0 ± 1.00	10.10	<0.001
Anal drags							
Number	8.4 ± 1.49	17.0 ± 2.31	20.2 ± 2.35	19.9 ± 1.42	22.1 ± 1.82	9.60	<0.001
Duration (sec)	18.8 ± 4.28	31.9 ± 6.53	39.6 ± 5.80	46.7 ± 8.00	42.5 ± 6.53	5.29	<0.001

The techniques used to collect headspace gas from the samples and to separate and identify the volatile components by gas chromatography (GC) and gas chromatography–mass spectrometry (GC-MS) are described in detail elsewhere (Goodrich et al., 1978, 1981, 1990). Volatiles were collected from feces by passing oxygen-free nitrogen (40 ml/min) over a 0.5-g sample for 30 min, from anal secretion by passing nitrogen over a 25-μl sample for 20 hr, and from urine by passing nitrogen over a 0.15-ml sample for 30 min. All samples were stored over solid carbon dioxide until required.

In addition to analyzing the anal secretion for volatile compounds, this material was tested for the presence of carbohydrate and protein (Feigl, 1966).

Results. Figure 2 shows the gas chromatograms of the headspace gases collected from fresh feces, anal secretion, and urine. Samples collected from both feces and anal secretion were rich in compounds with short retention times and many of these appeared to be common to the two materials. Samples from these two sources also contained compounds with long retention times, but the fecal sample contained more of these than the sample from anal secretion. These constituents with long retention times probably originated from the food remains in fecal pellets. Differences between the fecal and anal secretion samples in the total quantity of volatiles present were due, almost certainly, to differences in the quantity of the two materials available for analysis. Compounds with short retention times were less well represented in the headspace gas from the urine sample than in those of feces and anal secretions.

Most of the compounds identified in the headspace gas from the feces of BALB/c males were the same as those identified in the headspace of feces of wild mice (Goodrich et al., 1990) but some compounds were better represented and others less well represented in the BALB/c feces than in wild feces. One compound, 3-methylbutyl propanoate, which was found in aged feces from wild mice, was not detected in either fresh or aged feces from BALB/c mice.

Carbohydrates and proteins, both effective carries of volatile compounds, were detected in anal secretion, which suggests that this material is largely mucus from the tubular glands in the lower bowel.

Discussion. The similarities in the gas chromatograms of the headspace gas of fecal pellets and the headspace of anal secretion was not unexpected, for the fluid in feces is derived, largely, from the secretions of glands opening into the digestive tract, and fecal pellets are coated with mucus from the tubular glands in the wall of the rectum and with sebum from the anal glands before they pass to the exterior.

Experiment 3–Fecal Volatiles and Deposition of Feces and Urine

House mice housed singly usually deposit their urine in corners or at sites adjacent to walls (Desjardins et al., 1973) and their feces in the proximity of their nesting and feeding sites (Goodrich et al., 1990). These deposition pat-

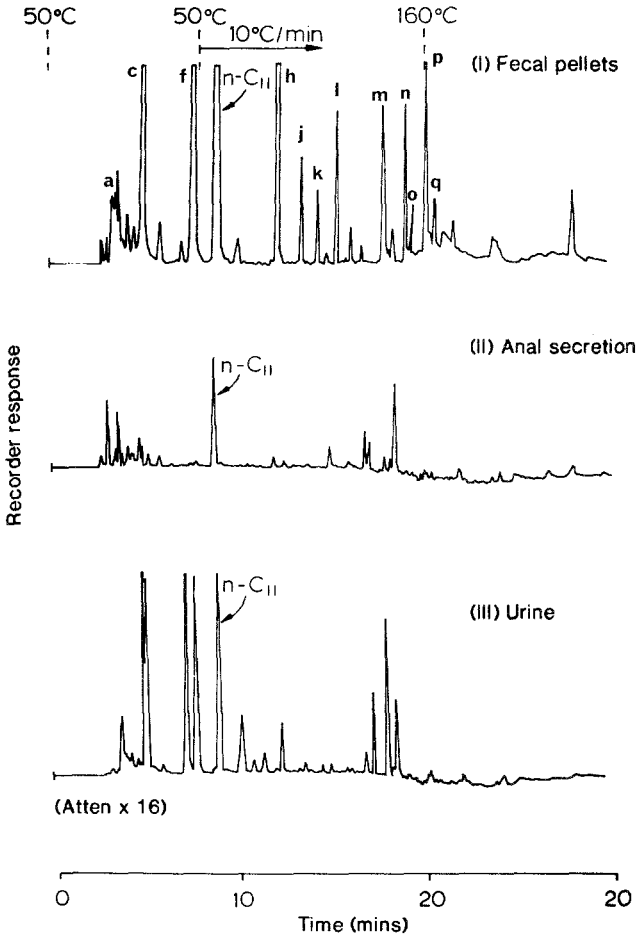


FIG. 2. Gas chromatographic comparison of the volatile constituents in headspace from fresh feces, anal secretion and urine from BALB/c male mice. Letters refer to compound indentifications shown in Table 1 (Goodrich et al. 1990).

terns are disturbed when strangers or their feces are introduced into an adjoining cage; the presence of a stranger causes the test mouse to deposit urine in small drops throughout its territory (Desjardins et al., 1973), and the presence of feces from strangers causes the animal to increase fecal deposition in the same vicinity (Goodrich et al. 1990). In this experiment we examined the effect of volatiles from the fecal pellets of strange conspecfics on the sites at which BALB/c males deposit urine and feces.

Methods. The test mice were 12 adult BALB/c males. Fecal samples to be used as a source of volatiles were collected by placing each of the test mice in

a cage with a wire mesh floor for 16 h. The pellets deposited on the blotting paper beneath the cages of the 11 animals not being used in the test were pooled and stored at −18°C until needed.

The apparatus and the procedure used to determine the pattern of urinary and fecal deposition are described in detail elsewhere (Goodrich et al., 1990). Briefly, the test mouse was housed for 16 h a day on two successive days in an arena (30 × 30 × 30 cm) fitted with a central shelter where food and water were freely available. The blotting paper beneath the wire mesh floor of the arena was divided into three equal sections (30 × 10 cm). On day 1 a stream of clean air (200 ml/min) was directed into the arena along the long side of section 3. On day 2 the airstream entering the arena along the long side of section 3 was passed over feces from strange males (2 g). The numbers of fecal pellets in each of the three sections beneath the arena were counted at the end of each day. The outlines of the urine spots were then visualized under ultraviolet light, their outlines traced, and their areas measured with the aid of a graphics tablet (Houston Instruments Hipad Digitizer) interfaced to an Apple II+ Computer.

The significance of the differences between the number of pellets (or the area of the urine spots) deposited on days 1 and 2 in each of the three sections was determined by applying chi-square tests to the data for each of the 12 mice.

Results. When the air flowing through the arena was clean, the test mice deposited over 70% of their fecal pellets and nearly 50% of their urine in the section beneath the shelter and the food and water supplies (area 2) (Table 2).

TABLE 2. MEAN NUMBER (±SE) OF FECAL PELLETS DEPOSITED BY BALB/c MALES (12) IN TEST ARENA OVER 16-HOUR PERIOD ON DAYS 1 AND 2 AND MEAN AREA (cm^2) OF BLOTTING PAPER BENEATH CAGE SOILED BY URINE, TOGETHER WITH MEAN PERCENTAGES OF FECAL PELLETS AND URINE DEPOSITED IN EACH OF THREE AREAS BENEATH ARENA[a]

		Mean percentage		
	Total	Area 1	Area 2	Area 3
Fecal pellets (number)				
Day 1	112 ± 11.3	16 ± 1.4	71 ± 1.4	13 ± 1.4
Day 2	125 ± 8.5	17 ± 1.2	29 ± 1.8	54 ± 1.7
Urine spots (area)				
Day 1	11 ± 2.2	18 ± 7.9	49 ± 8.4	33 ± 8.1
Day 2	13 ± 3.1	38 ± 7.7	33 ± 5.3	29 ± 7.7

[a] On day 1 clean air flowed into the arena along the outer margin of area 3; on day 2 the air flowing into the arena passed over fecal pellets (2 g) collected from males that were strangers to the test mice.

When the air flowing through the arena carried volatiles from the feces of strange males, the percentage of fecal pellets deposited by the test animals in area 2 decreased and the percentage in area 3 increased. The differences between the numbers deposited in the three areas on days 1 and 2 were significant for all 12 test mice (all chi-square values were greater than 11.0; $P < 0.001$). Urine deposition, on the other hand, did not appear to be affected by the presence of volatiles from the feces of strangers; in two cases the amount of urine in area 3 was greater on day 2 than on day 1 and the between-day difference was significant, but in six cases the between-day difference failed to reach significance and in four cases less urine was deposited in area 3 on day 2 than on day 1.

Discussion. In BALB/c mice, like wild mice (Goodrich et al., 1990), volatiles from the feces of strangers clearly affected the site at which the resident animal deposited its feces. Urine deposition, on the other hand, appeared to be unaffected by fecal volatiles.

Experiment 4—Volatiles from Feces and Anal Secretion and Outcome of Encounters between Conspecifics

A wild male, placed with a conspecific in an arena containing its own feces, usually initiated more aggressive attacks than its companion (Goodrich et al., 1990). The volatiles responsible for this increase in attack behavior on the part of the donor mouse could have been from the food remains or the anal secretions in the fecal pellets or from both sources. In this experiment we examined the effects of fecal pellets and of anal secretion alone on the outcome of social interactions between male BALB/c mice.

Methods. Thirty-two BALB/c males were used to test the effect of the presence of feces on the outcome of social interactions, and 20 males were used to test the effect of anal secretion. A week before the tests were carried out, the mice in both the feces group and the anal secretion group were divided into two subgroups (groups 1 and 2) that were housed in separate rooms. For each mouse of a given weight in group 1 there was a mouse of similar weight in group 2.

Feces were collected from each of the mice used in the fecal tests by housing it in a cage with a wire mesh floor kept overnight over a clean sheet of blotting paper. The pellets were collected the following morning, transferred to vials, and stored at $-18°C$. Anal secretion samples were collected by placing the mouse in a cage lined with clean aluminium foil for 1 h. The material deposited during this period was scraped from the foil with a small spatula, transferred to a vial, and stored at $-18°C$.

The arena and the procedure used to test the effects of feces and anal secretions on the outcome of social encounters is described in detail elsewhere (Goodrich et al., 1990). Briefly, the feces (0.5 g) or anal secretion (5 mg in 0.5

ml distilled water) of a group 1 mouse (the donor) was introduced into the arena where the test was to be conducted. The donor and a mouse of similar weight from the group 2 (the nondonor) were then introduced into the arena. After the two had made contact, their activities were observed over a period of 10 min and records were kept of the incidence and duration of the following activities: exploratory forays (locomotion about the test chamber), nonaggressive contacts (sniffing, grooming, nudging), and aggressive contacts (fighting, chasing). About two weeks later the test was repeated with the same pair of mice but with the feces (or anal secretion) of the group 2 mouse in the arena.

In both experiments all mice were tested twice, once in the presence of their own feces (donor situation) and once in the presence of the feces of their test partner (nondonor situation). For each of the behavioral activities recorded, means were calculated for the donor and the nondonor situation and means and variances for the donor–nondonor differences. To minimize the effects of between-mouse differences in activity, the variances were calculated for the sums of the donor–nondonor differences for each pair of mice. Single-sample t tests were used to determine the significance of the donor–nondonor difference for each activity. The tests were carried out on the transformed data using the formula:

$$\text{Log}_e\,[(\text{donor score} + 1)/(\text{nondonor score} + 1)]$$

Chi-square tests were used to test the significance of the effect of the presence of their own or their test partner's feces (or anal secretion) on the number of mice initiating aggressive interactions.

Results. In most encounters, the mouse that donated the feces placed in the arena appeared to be more successful than the nondonor, for the donor–nondonor difference was significant for both the number of nonaggressive contacts initiated and for the duration of those contacts (Table 3). Unlike wild males, only a few of the BALB/c males initiated aggressive contacts with their partners, but the number of contacts initiated by the donor males was significantly greater than the number initiated by nondonors (donors, 8/32, nondonors 1/32; chi square = 6.34, $P < 0.05$).

The donor mouse also appeared to have more success during encounters with a conspecific when its anal secretion was present in the arena, because the donor–nondonor difference was significant for both the number of exploratory forays undertaken and for the number of nonaggressive contacts initiated (Table 4).

Discussion. These results suggest that the presence of volatiles from their own feces or anal secretions improves the success rate of BALB/c males during social encounters with conspecifics. The results also suggest that the volatiles responsible for this effect are from the secretions present in the fecal pellets rather than from food remains.

TABLE 3. MEAN NUMBER AND MEAN DURATION (SEC) OF EXPLORATORY FORAYS, NONAGGRESSIVE CONTACTS, AND AGGRESSIVE CONTACTS MADE BY BALB/c MALE HOUSE MICE OVER 10-MINUTE PERIOD WHEN TWO MICE WERE PLACED IN TEST CHAMBER CONTAINING FECES OF ONE OF THEM (DONOR), TOGETHER WITH MEAN DIFFERENCE (±SD) BETWEEN SCORES WHEN TEST MOUSE WAS DONOR AND SCORES WHEN TEST MOUSE WAS NONDONOR

	Donor situation		Nondonor situation		Mean difference (±SD) (donor − nondonor)	Significance of difference	
	Occurrence	Mean score	Occurrence	Mean score		*t*	*P*
Exploratory forays							
Incidence	32	47.3	32	47.3	0.0 ± 8.9	0.20	NS
Duration		220.3		215.3	4.9 ± 95.7	0.29	NS
Nonaggressive contacts							
Incidence	32	50.4	32	39.4	11.1 ± 36.3	2.68	<0.05
Duration		182.2		138.0	44.2 ± 164.8	2.86	<0.05
Aggressive contacts							
Incidence	8	1.3	1	0.1	1.2 ± 4.9		
Duration		0.6		0.1	0.6 ± 2.3		

TABLE 4. MEAN DURATION (SEC) OF EXPLORATORY FORAYS, NONAGGRESSIVE CONTACTS, AND AGGRESSIVE CONTACTS MADE BY BALB/c MALE HOUSE MICE OVER 10-MINUTE PERIOD WHEN TWO MICE WERE PLACED IN TEST CHAMBER CONTAINING ANAL SECRETION OF ONE OF THEM (DONOR), TOGETHER WITH MEAN DIFFERENCE (±SD) BETWEEN SCORES WHEN MOUSE WAS DONOR AND SCORES WHEN IT WAS NONDONOR

	Donor situation		Nondonor situation		Mean difference (±SD) (donor − nondonor)	Significance of difference	
	Occurrence	Mean score	Occurrence	Mean score		*t*	*P*
Exploratory forays							
Incidence	20	40.8	20	29.8	11.0 ± 31.8	2.42	<0.05
Duration		190.8		252.0	−61.2 ± 300.6	−0.96	NS
Nonaggressive contacts							
Incidence	20	55.1	20	39.0	16.2 ± 40.5	2.63	<0.05
Duration		208.9		217.4	−8.6 ± 155.6	0.14	NS
Aggressive contacts							
Incidence	3	0.4	0		0.4 ± 1.7		
Duration		1.4			1.4 ± 8.2		

RESULTS AND DISCUSSION

For an excretory product to contribute to the recognition of territorial boundaries, it must be widely distributed and the volatiles from the product deposited by one mouse (or set of mice) must be qualitatively or quantitatively different from those from the product deposited by a second mouse (or set of mice). Both urine and feces are distributed widely in undisturbed habitats occupied by house mice (Welch, 1953; Pennycuik, 1973; Hurst, 1987).

Schwende et al. (1986) showed that urinary volatiles from mice of one strain may differ both quantitatively and qualitatively from those from mice of another. Our results (experiment 2), which show that fecal pellets include secretions from glands associated with the digestive tract as well as food remains, strongly suggest that volatiles from this source, too, may differ from strain to strain and from mouse to mouse. Feces as well as urine, therefore, could contribute to the volatiles that enable house mice from one group to distinguish the boundaries of their territory from those of neighboring groups.

The results of experiment 1, like those of other investigators (see Archer, 1975, for review), strongly suggest that mice deliberately deposit material from the anus when they are confronted with clean, unfamiliar surroundings (in familar surroundings both urine and feces accumulate at the sites where the animals spent most of their time). The results of experiments 3 and 4 suggest that the volatile compounds from the feces and anal secretions deposited by house mice, like those from their urine (see Brown, 1985, for review) may contribute to territorial recognition, for both the site at which the test animals deposit their feces and the outcome of encounters between conspecifics appear to be affected by the source of the feces and anal secretions to which they were exposed.

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INTERACTION BETWEEN VISUAL AND OLFACTORY STIMULI DURING HOST-FINDING BY LEAFHOPPER, *Dalbulus maidis* (HOMOPTERA: CICADELLIDAE)

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Abstract—Virtually nothing is known about the role plant volatiles play in host-finding by Homoptera in the Suborder Auchenorrhyncha. In laboratory bioassays, we examined the influence of plant volatiles on orientation and postcontact behaviors of the leafhopper, *Dalbulus maidis*, and determined the relationship between visual and olfactory stimuli during host-finding. When compared to the number of contacts made with reflected green light in the presence of a hexane control, *D. maidis* made more contacts when exposed to volatile extracts from its preferred host, maize; a similar number of contacts when exposed to volatiles from a marginal host, gamagrass; and fewer contacts when exposed to volatiles from a nonhost, sorghum. There was no difference between males and females in the number of contacts made with green light when exposed to maize volatiles compared to hexane alone. More contacts were made with green light than with white light of similar intensity, both in the presence and in the absence of olfactory stimuli; however, maize volatiles acted as a synergist by increasing the number of contacts leafhoppers made with green light. After contacting the green light, exposure of maize volatiles significantly increased, relative to hexane, the amount of stationary time, but did not influence the amount of time spent moving, the distance traveled, or the speed while moving when within the boundaries of the green light. This study provides the first evidence for an interaction between visual and olfactory stimuli during host-finding for a leafhopper and also for olfactory mediation of postcontact behaviors not associated with feeding.

Key Words—Leafhopper, *Dalbulus maidis*, Homoptera, Cicadellidae, host-finding, maize, visual, olfactory, synergism, pest.

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INTRODUCTION

Current research suggests differences exist between the two suborders of Homoptera, the Sternorrhyncha and the Auchenorrhyncha, in the importance of volatile chemicals during intraspecific communication and host-finding, with the Sternorrhyncha (aphids, whiteflies, mealybugs, psyllids) being much more responsive to odors than the Auchenorrhyncha (leafhoppers, planthoppers, treehoppers, spittlebugs, cicadas). Olfactory perception of alarm pheromones (Nault and Phelan, 1984, and references therein) and sex pheromones (Cardé and Baker, 1984, and references therein; Eisenbach and Mittler, 1987) has been demonstrated for several Sternorrhyncha. Olfactory receptors responsive to plant volatiles have been identified from aphids (Bromley and Anderson, 1982; Yan and Visser, 1982), and several aphid species are known to alter their behavior when exposed to host and nonhost odors in the laboratory (Pettersson, 1970; Tamaki et al., 1970; Visser and Taanman, 1987) and in the field (Chapman et al., 1981). In contrast, intraspecific communication within the Auchenorrhyncha is mediated primarily by acoustic signals (Claridge, 1985; Heady et al., 1986), although some treehoppers respond to alarm pheromones (Nault et al., 1974). Only a few Auchenorrhyncha have been shown to possess host odor receptors (Klein et al., 1988) and to respond to plant volatiles during host-finding (Saxena and Saxena, 1974; Khan et al., 1988).

The Neotropical leafhopper genus *Dalbulus* is composed of 11 species that utilize maize, *Zea mays* L., and its wild relatives, the teosintes (*Zea* species) and the gamagrasses (*Tripsacum* species) as hosts (Nault, 1985). One species, *Dalbulus maidis* (DeLong & Wolcott), is a maize specialist and considered a serious pest in Latin America, primarily by transmitting maize-stunting pathogens (Nault, 1990). Virtually nothing is known about host-finding behaviors of *Dalbulus* leafhoppers. Todd et al. (1990a) examined the importance of color stimuli in host-finding by three *Dalbulus* species by comparing pre- and postcontact responses to maize seedlings with those to vertical models differing in hue (dominant wavelengths) or value (total amount of light reflected). Leafhoppers exhibited a strong orientation response to yellow and green models, and maintained contact with these colored models as long or longer than models painted other colors. However, the models provided only color and perhaps structural stimuli and did not elicit contacts by as many leafhoppers as maize. Tenure after contact also was significantly shorter on models than on maize. These data suggested that additional plant stimuli were used by *Dalbulus* leafhoppers during host-finding and/or accepting behaviors. After contacting a plant, most leafhoppers can detect external plant chemicals during surface exploration, and internal plant chemicals during probing and ingestion (Backus, 1985). However, only two species of *Empoasca* leafhoppers have been shown to respond to olfactory plant stimuli prior to contact (Saxena et al., 1974). We

suggest that the specialization and pest status of *D. maidis* on maize may be enhanced if leafhoppers can differentiate maize from other plants while airborne or before initiating feeding. Therefore, in a series of laboratory bioassays, we examined the influence of plant volatiles on orientation and postcontact behaviors of male and female *D. maidis* and determined the relationship between visual and olfactory stimuli during host-finding.

METHODS AND MATERIALS

Leafhoppers. Leafhoppers used in bioassays were between one and four weeks post-adult eclosion and were obtained from colonies kept in a rearing room maintained at 26 ± 2°C and a 14:10 light–dark photoperiod. Colonies were established from individuals originally collected in Mexico (Nault, 1985), and maintained on six-leaf maize (cv. Aristogold Bantam Evergreen) for 20–60 generations in the laboratory. Leafhoppers were starved for 5–7 hr prior to testing to predispose them to host-finding (Todd et al., 1990) by releasing them into a plywood transfer hood (Heady and Nault, 1985). For bioassays requiring sexed adults, leafhoppers were anesthetized with CO_2, and males and females were segregated into 30-cm × 7.2-cm-diam. butyrate tubes. The tubes were placed in the transfer hood until leafhoppers were conditioned for bioassays.

Source of Volatiles. Extracts from the whorl leaves of three four-leaf maize seedlings were used as a source of volatiles for most bioassays. Whorl tissue was chosen because adults of *D. maidis* are found in high numbers in maize whorls in the field (Power, 1987) and in greenhouse studies (Todd et al., 1990b). Seedlings were cut just below the whorl. The whorl leaves were then placed in a glass tissue grinder and macerated in 30 ml of HPLC-grade hexane for 15 min. Extracts also were obtained from gamagrass, *Tripsacum dactyloides* (L.)L., and seedling sorghum, *Sorghum bicolor* (L.) Moench. Gamagrass leaves of roughly similar age to maize leaves were obtained by removing the youngest basal leaves that had not completely unfurled, and sorghum leaves were obtained in the same manner as maize leaves. All extracts were stored at −10°C prior to use in bioassays.

Observation Chamber. Bioassays were conducted in a Plexiglas observation chamber (OC) spray-painted black (Dutch Boy Flat Black 3727, The Sherwin-Williams Co.) on all outer surfaces except the top, which was left clear to facilitate observations (Figure 1). The side panels were removable. Two 5-cm × 5-cm windows were cut in the front panel to hold a 550-nm narrow band interference filter (green foliage stimulus) or neutral density filters (Oriel Corp.) of the same dimensions that served as the source of visual stimuli. The windows were centered on the panel and spaced 5 cm apart. The chamber was located in a dark room. A Kodak Ectagraphic III slide projector equipped with a 300-W

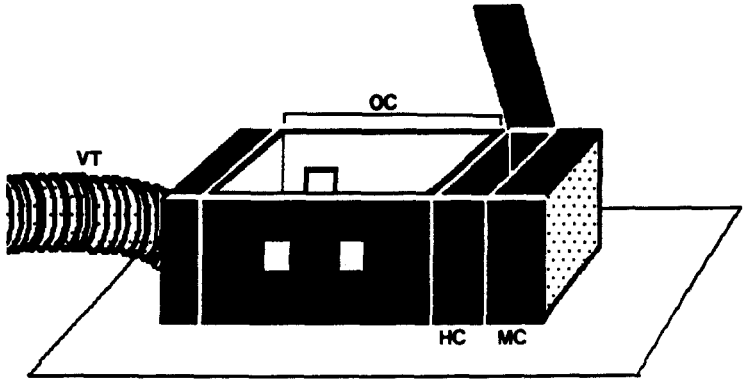


FIG. 1. Observation chamber for testing the response of *D. maidis* to plant extracts. OC = observation chamber; MC = mixing chamber; HC = holding chamber; VT = vacuum tubing. The direction of airflow was from the right (upwind) to the left (downwind). Leafhopper contacts with a reflected square of light were recorded by transmitting projector light through filter(s) placed in the downwind window. Chamber dimensions are 25.5 × 21 × 19 cm.

quartz projector bulb was placed 60 cm in front of the chamber, and the projector light was transmitted through the filter(s) so a reflected image appeared on the opposite chamber wall.

To determine the effects of volatiles on leafhopper host-finding behavior, the chamber was modified by replacing the side panels with nylon mesh screening and by adding a mixing chamber (MC) to stabilize airflow and a holding chamber (HC) for the volatile source at the upwind end of the original chamber (Figure 1). The holding chamber was constructed from 8-cm × 10-cm × 1-cm pieces of wood painted black and placed next to the screening. The top of the holding chamber was removable. The mixing chamber was the same dimensions as the holding chamber and was wrapped tightly with three layers of fine cheesecloth. The two chambers were attached to each other and to the original chamber using black duct tape. An exhaust fan was used to pull volatiles through the chamber at a rate of 2.5 cm/sec and into 10-cm-diam. vacuum tubing (VT) attached to a separate box located at the downwind end of the chamber (Figure 1). The movement of the odor plumes was estimated using titanium tetrachloride and was observed to be nonlaminar, with considerable mixing throughout the chamber.

General Bioassay Procedures. Twenty leafhoppers were introduced simultaneously, unless otherwise specified, 1 min prior to volatile presentation at the downwind end of the chamber. Leafhoppers were released by gently tapping them from an aspirator, which was positioned 2–3 cm above the center of the chamber floor. A volatile source was provided by pipetting 1 ml of maize,

gamagrass, or sorghum extract (0.1 plant equivalents) onto 5.5-cm-diam. filter paper that was attached with an alligator clip to a piece of wire in a rubber cork; the height at the middle of the filter paper was 10 cm. The control consisted of filter paper treated with 1 ml of hexane. The volatile source was placed in the holding chamber (Figure 1), and data were collected on the number of contacts leafhoppers made with a reflected green square of light (bioassays 1–3) produced by transmitting the projector light through the 550-nm narrow band interference filter. Leafhopper response to reflected rather than transmitted light was examined based on previous knowledge of *Dalbulus* host-finding behavior (Todd et al., 1990a) and on observations indicating that fewer contacts were made with the actual filter than with the reflected image. A contact was recorded if an individual walked or flew directly onto the green light or turned back into the light after leaving momentarily. The number of contacts was recorded every minute for 15 min. Bioassays were conducted between 6 and 12 hr after lights on (14:10 light–dark photoperiod) and replicated 10 times unless otherwise specified, with a replicate consisting of a treatment and control run sequentially. A different group of 20 leafhoppers was used for each treatment and control, and after a bioassay, leafhoppers were returned to colonies. Preliminary observations indicated the window position of the filter did not elicit differential leafhopper contacts; therefore, all bioassays were conducted with the filter placed in the downwind window. The upwind window was covered temporarily with a piece of black construction paper until needed in bioassay 4 to examine the postcontact responses of leafhoppers to green light in the presence and in the absence of olfactory stimuli. All bioassays included a visual stimulus because leafhoppers released into the chamber with only chemical stimuli did not move off the chamber floor and exhibited no upwind orientation.

Bioassay 1. Response to Green Light in Presence of Plant Volatiles. Orientation of *D. maidis* to green light in the presence of volatiles from maize, gamagrass, and sorghum was quantified separately as described in the general bioassay procedures.

Bioassay 2. Response to Green Light by Males and Females in Presence of Maize Volatiles. Differences between the sexes in orientation to green light in the presence of maize volatiles were determined using a randomized complete block design ($N = 10$) with four treatments presented separately: male + hexane; male + extract; female + hexane; and female + extract. One block was conducted per day, with 20 individuals used for each treatment within a block.

Bioassay 3. Relationship between Light Quality and Maize Volatiles. The relationship between visual and olfactory stimuli during the orientation phase of host-finding by *D. maidis* was determined by quantifying the number of contacts made with two visual stimuli, represented either by green light or by white light, in the presence and in the absence of maize volatiles. The intensity of the white light was made similar to that of the green light (2 $\mu E/m^2/sec$) by placing

0.1, 0.3, and 0.6 neutral density filters in the downwind window. Light intensity was measured using a portable LI-185B Photometer (Li-Cor, Inc., Lincoln, Nebraska). A randomized complete block design ($N = 10$) was used with four treatments presented separately: green light + hexane; green light + extract; white light + hexane; and white light + extract. One block was conducted per day, with 20 individuals used for each treatment within a block.

Bioassay 4. Postcontact Responses to Green Light in Presence of Maize Volatiles. To examine the chemically modulated, postcontact behaviors of *D. maidis* to green light, a piece of graph paper with 0.5-cm^2 grids was photocopied onto a transparency, and the transparency was taped to the back wall of the chamber where the green light appeared. To aid in detecting leafhoppers walking towards the light, the grid extended 3 cm in each direction beyond the lighted area. The grid lines outside of the light were illuminated by a 25-W incandescent red bulb positioned 10 cm above the chamber. A sheet of nonglare glass with the same inner dimensions as the chamber was placed in front of the grid to provide a smooth surface similar to that of the chamber walls. Leafhopper behavior was recorded using a RCA videocamera (model TC1005/U9) and a Panasonic videocassette recorder NV-8950. The camera was positioned so it viewed the grid through the upwind window on the front panel of the chamber. Twenty leafhoppers were observed individually for both the treatment and the control. Data were recorded on overall tenure within the green light, which was further subdivided into time spent stationary and time spent moving. The distance traveled was recorded by tracing the paths of individuals on an identical grid sheet photocopied onto a piece of paper. Distance (cm) was determined using an Inch Counter, an instrument that measures the length of a line or curve. Two measures of walking speed were determined by dividing the distance traveled by either the time spent moving or by the overall tenure, which included both stationary and moving time (overall speed).

Statistical Analyses. Homogeneity of variances was tested using Bartlett's test (Sokal and Rohlf, 1981). Data from bioassays 1 and 4 were log $(x + 1)$ transformed before being subjected to a t test for paired comparisons ($P \leq 0.05$), or a two-way analysis of variance (ANOVA) ($P \leq 0.05$) (bioassays 2, 3).

RESULTS

Bioassay 1. Response to Green Light in Presence of Plant Volatiles. D. maidis made approximately two times as many contacts with green light when exposed to maize volatiles as compared to hexane alone ($t = 3.69$, $df = 9$, $P < 0.01$) (Table 1). In contrast, the presence of gamagrass volatiles did not influence the number of contacts leafhoppers made with green light ($t = 0.235$,

$df = 9, P > 0.50$). Fewer contacts were made with the light when leafhoppers were exposed to sorghum volatiles compared to hexane alone ($t = 3.12$, $df = 9, P < 0.02$). When maize volatiles were present, more than half of the leafhoppers were observed moving towards the green light within 2–3 min after release into the chamber compared to 7–8 min in controls. Most leafhoppers oriented towards the light by walking across the chamber floor until they were against the wall–floor juncture and then proceeded to walk up the wall and onto the light. A few individuals flew directly onto the light.

Bioassay 2. Response to Green Light by Male and Female Leafhoppers in Presence of Host Volatiles. Both sexes made more contacts with green light when maize volatiles were present compared to hexane alone ($F = 6.68$, $df = 1,36$, $P < 0.025$). However, there was no significant difference in the number of contacts with green light made by males as compared to those made by females ($F = 2.72$, $df = 1,36$ $P > 0.10$) (Table 1), nor was there an interaction between sex and maize volatiles ($F = 0.513$, $df = 1,36$, $P > 0.25$). The mean number of contacts made with green light by males and females collectively was significantly higher when maize volatiles were present compared to hexane alone (Table 1).

Bioassay 3. Relationship between Light Quality and Maize Volatiles. The quality of reflected light (green vs. white) had a highly significant effect on leafhopper contacts (Table 1). In the absence of maize volatiles, leafhoppers consistently made more contacts with green light than with white light ($F =$

TABLE 1. INFLUENCE OF PLANT EXTRACTS ON CONTACTS MADE BY *D. maidis* WITH REFLECTED SQUARES OF LIGHT

Bioassay[a]	Sex	Plant extract[b]	$\bar{X}$ (±SE) of contacts with green light		$\bar{X}$ (±SE) of contacts with white light	
			Extract	Control	Extract	Control
1	mixed	M	33.1 ± 4.1a	17.2 ± 3.3b		
		G	12.4 ± 1.1a	12.9 ± 1.9a		
		S	3.9 ± 0.7a	8.8 ± 1.7b		
2	male	M	32.2 ± 6.7a	16.3 ± 3.4b		
	female		20.8 ± 5.3a	11.8 ± 2.5b		
	mean		26.5 ± 4.4a	14.1 ± 2.1b		
3	mixed	M	15.7 ± 2.0a	8.9 ± 1.0b	3.2 ± 0.9c	2.8 ± 0.8c

[a] For bioassay 1, means within a row followed by the same letter are not significantly different at $P \leq 0.05$ (t test for paired comparisons). For bioassays 2 and 3, means followed by the same letter are not significantly different at $P \leq 0.05$ by LSD.

[b] M = maize, G = gamagrass, S = sorghum.

37.9, $df = 1,36$, $P < 0.001$). The effect of chemical alone was not significant ($F = 3.95$, $df = 1,36$, $P > 0.05$). However, a significant interaction existed between visual and olfactory stimuli ($F = 5.31$, $df = 1,36$, $P < 0.05$), with exposure to maize volatiles increasing the number of contacts made with green light but not with white light of similar intensity.

Bioassay 4. Postcontact Responses to Green Light in Presence of Maize Volatiles. Regardless of whether stationary or moving, individuals spent more time inside the boundaries of the green light in the presence of maize volatiles than in their absence ($t = 3.47$, $df = 19$, $P < 0.01$) (Table 2). When overall tenure was subdivided into stationary time and moving time, significantly more time was spent stationary when individuals were exposed to maize volatiles compared to hexane alone ($t = 3.70$, $df = 19$, $P < 0.01$) (Table 2). Maize volatiles did not have an effect on the time spent in movement ($t = 0.077$, $df = 19$, $P > 0.90$), the distance traveled within the green light ($t = 0.494$, $df = 19$, $P > 0.50$), or the rate of locomotion when walking ($t = 1.07$, $df = 19$, $P > 0.20$) (Table 2). However, the overall rate of locomotion was significantly slower within the green light when maize volatiles were present compared to hexane alone, due to increased stationary time ($t = 3.09$, $df = 19$, $P < 0.01$) (Table 2).

DISCUSSION

Host-finding is generally a catenary process during which an insect can be influenced by both noncontact and contact plant stimuli (Miller and Strickler, 1984). Orientation and settling can be mediated by both visual and olfactory stimuli (Prokopy and Owens, 1983; Visser, 1986). Sustained contact may

TABLE 2. POSTCONTACT RESPONSES OF *D. maidis* TO GREEN LIGHT WHEN EXPOSED TO MAIZE VOLATILES

	$\bar{X}$ (±SE)	
Postcontact response	Maize extract	Control
Overall tenure (sec)	164.5 ± 25.9a[a]	75.1 ± 12.1b
Tenure stationary (sec)	155.9 ± 24.8a	66.7 ± 11.6b
Tenure moving (sec)	8.6 ± 1.8a	8.4 ± 1.4a
Distance traveled (cm)	10.2 ± 2.1a	10.1 ± 1.8a
Speed moving (cm/sec)	1.1 ± 0.2a	1.3 ± 0.2a
Overall speed (cm/sec)	0.07 ± 0 .01a	0.15 ± 0.02b

[a] Means within a row followed by the same letter are not significantly different at $P \leq 0.05$ (t test for paired comparisons).

require additional mechanical and/or gustatory stimuli, which presumably provide the insect with more specific information concerning suitability of the plant for feeding or oviposition (Scriber and Slansky, 1981). In the present study, we showed that olfactory plant stimuli influenced both orientation and postcontact behaviors of *D. maidis* to a foliage stimulus (Tables 1 and 2). Exposure to maize volatiles approximately doubled the number of contacts leafhoppers made with green light compared to contacts made with green light in the presence of hexane alone (Table 1). Discrepancies in the number of contacts made with green light in bioassays 1–3 may be due to differences in the flight activity of leafhoppers that were tested at different times during their photoperiod (R.A.J. Taylor, unpublished data). Volatiles from whorl tissue (Thompson et al., 1974) and other maize structures (Buttery and Ling, 1984) consist mainly of six-carbon green leaf-type compounds and sesquiterpenes, compounds that are attractive to many phytophagous insects during host-finding (Visser, 1986). Similar volatiles were identified from the homogenized-leaf maize extract used in this study and from the headspace over intact leaves using GC-MS (Todd and Phelan, unpublished data).

D. maidis also exhibited a neutral response to gamagrass volatiles, and a negative response to sorghum volatiles (Table 1, bioassay 1). Although the constituents of gamagrass odor are not known, the lack of a significant response of *D. maidis* to odors from this plant compared to maize suggests that gamagrass volatiles are either qualitatively or quantitatively different from those of maize. *D. maidis* has been collected from gamagrass only when maize in nearby fields has died back (Triplehorn and Nault, 1985). In the laboratory, *D. maidis* reproduces on gamagrass, but the progeny are few in number, small in size, and take longer to develop than those on maize (Nault and Madden, 1985). Therefore, a weak response to gamagrass odor in the field may limit contacts, and thereby prevent some females from ovipositing in a plant that is not optimal for nymphal development and population growth. A chemical basis for resistance of sorghum to feeding by some homopterans may be due to HCN compounds and various phenolic acids (Dreyer et al., 1981). Although maize and sorghum look the same during seedling stages, if sorghum volatiles act as repellents, they may provide a mechanism by which *D. maidis* avoids sorghum. The nonhost status of sorghum for *D. maidis* has been established in the laboratory (L.R. Nault, unpublished data).

Perhaps even more interesting than determining responsiveness of *D. maidis* to olfactory plant stimuli was determining that there was a significant interaction between host volatiles and the quality of reflected light (Table 1, bioassay 3), with maize volatiles acting as a synergist by increasing the number of contacts *D. maidis* made with green light, but not with quantitatively similar white light (Table 1). Chapman et al. (1981) also provided evidence for an interaction between visual and olfactory stimuli during host-finding by the aphid,

Cavariella aegopodii (Scopoli), which was trapped in higher numbers in yellow water traps baited with carvone compared to unbaited yellow traps or colorless traps. These two studies provide the only documentation of an interaction between visual and olfactory stimuli within the Homoptera. In the field, the responsiveness of *D. maidis* to green reflected light may aid in distinguishing foliage from nonfoliage. However, because most plants are green, the use of color stimuli alone during host-finding may result in leafhoppers making contacts with nonhosts. The present data suggest that close-range chemoreception of plant volatiles ($\leq$20 cm from a source), coupled with an attractive color, may enable *D. maidis* to distinguish maize from nonhost plants while airborne (Table 1, bioassay 3). Chemoreception over a longer distance may be possible but could not be determined in our bioassays. There were no differences between the sexes in orientation to green light when exposed to maize volatiles, compared to hexane alone (Table 1, bioassay 2). Responsiveness to host odors by both male and female *D. maidis* may aid in bringing the sexes together for mating and has been shown for several Lepidoptera (Hansson et al., 1989, and references therein).

The influence of host odors on the postcontact, nonfeeding behaviors of leafhoppers had not been documented prior to this study, which provides the first evidence that volatiles from a host can increase leafhopper tenure (Table 2). This observed increase in tenure was due to individuals spending more time stationary on the green light in the presence of maize volatiles than in the presence of hexane alone (Table 2), perhaps because the volatiles stimulated *D. maidis* to initiate probing. That probing is initiated soon after contact with maize seedlings is suggested by electronic monitoring of feeding behavior (A.C. Wayadande, unpublished data) and by observations that *D. maidis* remains at or near its initial landing position on seedling maize for up to 3 hr (Todd et al., 1990a). By comparison, exposure to maize volatiles resulted in a mean tenure of only 2.75 min for leafhoppers on green light (Table 2), suggesting that gustatory and/or mechanical stimuli perceived during feeding are of primary importance in maintaining leafhopper contact with a host.

Although postcontact behaviors of the sexes were not observed separately, observations suggested that males spent more time moving within the boundaries of the green light than females in both the treatment and the control. Greater male mobility was demonstrated for *Dalbulus* species by Heady and Nault (1985), who showed that males were more likely to fly when mechanically dislodged from maize seedlings than females. Hunt (1988) found that male *Graminella nigrifrons* (Forbes) leafhoppers engaged in more interplant flights than females when given access to oat seedlings (*Avena sativa* L.), a strategy for increasing the likelihood of locating a calling virgin female. Similarly, if *D. maidis* males are very mobile after contacting a host plant, they may increase

their likelihood of locating females during intraplant movements and thereby enhance their mating success.

The present data, coupled with previous knowledge on the responses of *D. maidis* to color stimuli (Todd et al., 1990a), provide a more complete understanding of host-finding behaviors of this species. The strong orientation response of *D. maidis* to yellow and green may aid this leafhopper in distinguishing foliage from nonfoliage when flying above plant canopies. When combined with an attractive color, the differential responsiveness of *D. maidis* to plant odor may further aid it in orientation and settling. In addition, we have provided further insight into the specialization and pest status of *D. maidis* on maize by demonstrating that maize volatiles not only have an effect on orientation, but also on postcontact behaviors not associated with feeding.

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CHEMISTRY OF MALE MANDIBULAR GLAND SECRETIONS OF *Philanthus triangulum*

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Abstract—Males of the European and African beewolf, *Philanthus triangulum*, possess a sex specific mandibular gland secretion that is used for marking plant stems in mating territories. The secretion is composed of 90% (*Z*)-11-eicosen-1-ol plus small amounts of 10-nonadecen-2-one, nonadecenal, octadecanoic and octadecenoic acids, and eicosenal. The chemistry of this secretion differs markedly from the secretions of North American *Philanthus*, which consist of a larger number of components that possess different chemical functionalities and are more volatile. We postulate that the chemical differences between *P. triangulum* and its New World relatives reflect phylogenetic differences plus a possible reduced necessity for species isolating mechanisms in *P. triangulum*.

Key Words—*Philanthus triangulum*, Hymenoptera, Sphecidae, Philanthinae, beewolf, (*Z*)-11-eicosen-1-ol, 10-nonadecen-2-one, nonadecenal, eicosenal, pheromone.

INTRODUCTION

Beewolves in the genus *Philanthus* are a large widely distributed group of sphecid wasps found throughout much of the Old World and North America. One species, *Philanthus triangulum* (F.), which is often simply called the beewolf, is a common European and African wasp that preys only on honeybees (*Apis mellifera*) and can be a serious pest of apiculture (Simon Thomas and Simon Thomas, 1980). The behavior of this common European wasp has been studied in depth by a variety of investigators who elucidated the mechanisms of prey

and nest-site location and orientation (summarized in Tinbergen, 1972), recorded nesting and mating behavior (Simon Thomas and Poorter, 1972) and studied the stinging behavior of *P. triangulum* and the paralyzing properties of the venom (Rathmayer, 1962). The pharmacological activity of this venom is probably better understood than that of any other solitary wasp species (Piek and Mantel, 1986, and references therein). An excellent overview of the biology of *P. triangulum* and other beewolves is provided by Evans and O'Neill (1988).

The mating behavior of *Philanthus*, including *P. triangulum*, is unusual among sphecids and other wasps in that males have scent-marked territories that they defend (Simon Thomas and Poorter, 1972; Alcock, 1975; Gwynne, 1978; O'Neill, 1979, 1983; Borg-Karlson and Tengö, 1980). It is in these areas that most matings appear to occur. In parts of North America, several species of *Philanthus* are sympatric, sometimes even with temporal and spatial overlapping of male territories (Schmidt et al., 1985; Evans and O'Neill, 1988). In much of Europe, *P. triangulum* is the major species and, hence, interspecific interactions with other *Philanthus* are minimal.

At territory sites, males mark blades of grass and other parts of vegetation with mandibular gland secretions (Simon Thomas and Poorter, 1972; Alcock, 1975; Gwynne, 1978; O'Neill, 1979, 1983; Borg-Karlson and Tengö, 1980; Evans, 1983; Schmidt et al., 1985). These secretions are used as pheromones to attract females. In the North American species that have been studied, these pheromones are present in the heads of males but are absent in females and male thoraces and abdomens (Schmidt et al., 1985). The pheromones of these North American *Philanthus* are species-specific (Schmidt et al., 1985; McDaniel et al., 1987; unpublished data), and plants marked by males contain the same chemical compounds found in the males' mandibular glands (Schmidt et al., 1985).

Little is known of the chemical ecology of the pheromones produced by territorial males of *P. triangulum*. In an investigation specifically tailored to detect pyrazines in wasps, *P. triangulum* was discovered to contain trace quantities of alkylpyrazines (Borg-Karlson and Tengö, 1980). These compounds are probably not components of the sex pheromone, however, because (1) they are present in both males and females in approximately equal quantities, (2) are present in much smaller quantities than the ones we describe here, and (3) because pyrazines are widespread in wasps and other Hymenoptera (Blum, 1981) and have been demonstrated to act as common aposematic olfactory warnings (Guilford et al., 1987).

We investigated *P. triangulum* because this species inhabits a different geographic region (Europe and Africa) than the previously studied North American species and because its chemical ecology, unlike that of the North Amer-

ican species, should not be influenced in Europe at present by the necessity of species-isolating mechanisms. We report here the chemistry of the mandibular gland volatiles of this species.

METHODS AND MATERIALS

Primary analyses were performed on mandibular glands obtained as previously reported (Schmidt et al., 1985) from wasps collected in France, south of Bordeaux in Department Landes during August 1983. Typically, methylene chloride (Burdick and Jackson Laboratories, Inc., Muskegon, Michigan[4]) extracts of pooled heads, thoraces, and abdomens of male and female *P. triangulum* were used for analyses. For gas chromatographic (GC) analyses, the extracts were then taken to incipient dryness with a gentle stream of nitrogen and redissolved in ca. 2 ml hexane (UV grade, Mallinkrodt Inc., St. Louis, Missouri). The hexane extract was partitioned with 2 ml 6 N Na_2CO_3 solution; the hexane layer was removed, dried over anhydrous sodium sulfate, and placed on a mini-column of Bio Sil A (Bio-Rad Laboratories, Richmond, California). The hydrocarbons were eluted with 3 ml hexane, and the more polar compounds were removed with 3 ml tetrahydrofuran (UV grade, Mallinkrodt). Further separations of the components were achieved by spotting the THF extract on a TLC plate (Silica Gel F_{254}, E. Merck AG, Darmstadt, Germany) using a reagent containing rhodamine B and dichlorofluorescin as described by Jones et al. (1966).

Derivatives were prepared in Reactivials (Pierce Chemical Company, Rockford, Illinois) and heated in a Reactitherm unit. *N,N*-Dimethylhydrazones were prepared as described by McDaniel and Howard (1985). Double-bond positions were determined by methylthiolation followed by GC–mass spectrometry (GC-MS) (Francis and Veland, 1981). Double-bond stereochemistry and functional analyses of the primary component were determined by infrared (IR) spectroscopy on a Nicolet DX_5 after TLC separation was achieved. (*E*)-11-Eicosen-1-ol and (*Z*)-11-eicosen-1-ol were purchased from Nu-Chek-Prep, Inc., Elysian, Minnesota.

Acetate esters were prepared by placing microgram amounts of (*E*)- and (*Z*)-11-eicosen-1-ol isomers or the neutral lipid fraction into a 5-ml Reactivial and removing the solvent with a gentle stream of nitrogen. Approximately 1 ml of acetic anhydride and 0.1 ml pyridine were added. The mixture was held at 40°C for 4 hr, diluted to 20 ml with hexane, and washed three times by shaking

[4]Mention of a company, trade name or commercial product is for identification purposes only and does not imply endorsement by the USDA to the exclusion of others.

in a separatory funnel with 20-ml portions of 2 M $NaHCO_3$. The bottom aqueous layers were discarded. The hexane layer was dried over anhydrous Na_2SO_4 and the volume reduced in vacuo to a final volume of 2.0 ml.

Male *P. triangulum* in alcohol were used as a secondary source of biological material to enable the determination of the double-bond position of 10-nonadecen-2-one. This was achieved first by making the dimethyldisulfide (DMDS) derivatives, which were analyzed by mass spectrometry (Scribe et al., 1988; Buser et al., 1983; Dunkelblum et al., 1985; Francis and Veland, 1981), followed by derivatization with *N,N*-dimethylhydrazine and mass spectral analysis of the resultant *N,N*-dimethylhydrozone.

The GC-MS analyses were performed on two different systems: the first system was a Hewlett-Packard 5882 mass spectrometer interfaced to a Hewlett-Packard 5710A gas chromatograph with a 5933A Data System. The column employed was 1.8 m × 2 mm ID packed with 3% OV-101 on 100/120 mesh Ultrabond. The GC-MS parameters were: injection port temperature 250°C; oven temperature 150–280°C at 8°C/min; transfer line temperature 280°C; He at 13 ml/min; mass spectra were run at 70eV.

Later analyses were achieved with a Hewlett-Packard 5890A gas chromatograph with a 5970 Mass Selective Detector. The column employed was FSOT DB-5 (J&W Scientific, Folsom, California) 30 m × 0.25 mm. Analyses used temperature programmed from 120 to 300°C at 5°C/min.

RESULTS

Initial gas chromatographic analyses of the *P. triangulum* mandibular glands showed the presence of 11 components. Six of these components, including three hydrocarbons, were common to the abdomen, thorax, and head extracts of both male and female wasps; these were not examined further.

The principal component of this mixture was (Z)-11-eicosen-1-ol, which comprised approximately 90% of the total extract (Table 1). The mass spectrum of the compound yielded no molecular ion, but there is an M-18 ion at m/z =

TABLE 1. COMPOSITION OF *Philanthus triangulum* GLANDS

Component	Composition (%)
(Z)-11-Eicosen-1-ol	89.46
10-Nonadecen-2-one	5.17
Δ^x-Octadecenoic + octadecanoic acid	3.68
Δ^x-Nonadecenal	1.69
Δ^x-Eicosenal	tr

278 in 3% relative abundance. This compound, after isolation by preparative TLC, exhibited a broad absorption at 3482 cm^{-1}, corresponding to O—H stretch. Its respective absorption at 1060 cm^{-1} further indicated that the alcohol was primary or secondary. Oxidation of this compound yielded an acid, which established that the alcohol moiety was primary. Methylthiolation followed by GC-MS analysis established that the double bond was Δ^{11} and the absorption at 721 cm^{-1} suggested a *Z* double-bond configuration. The *Z* double-bond configuration was confirmed by coinjecting the acetate ester with acetate esters of authentic compounds. It was necessary to prepare acetate esters because separation could not be achieved between the underivatized alcohol isomers. Likewise, mass spectra of both isomers of the alcohol were identical, as were the mass spectra of both acetate esters. Baseline separation was achieved between the acetates with the *Z* isomer eluting first.

The second most abundant component of the male *P. triangulum* comprised 5.2% of the total secretion and was identified as 10-nonadecen-2-one. Diagnostic ions from the mass spectrum of the compound and its derivatives are listed in Table 2. The position of the unsaturation could not be determined from the dimethylhydrazone derivative. Treatment of the nonadecenone with dimethyldisulfide yielded a product whose diagnostic ions at 173 and 201 (*m/z*) indicated the unsaturation to be at either carbon 8 or carbon 10. Derivatiza-

TABLE 2. DIAGNOSTIC IONS OF SOME MANDIBULAR COMPONENTS AND THEIR DERIVATIVES OF *Philanthus triangulum*

Component/derivatives	Diagnostic ions (*m/z*)
(*Z*)-11-Eicosen-1-ol	82, 96, 278 (M-18)
Dithiomethyl ether of (*Z*)-11-eicosen-1-ol	173 (43%), 215 (26%), 217 (16%)
Methyl ester of oxidized 11-eicosen-1-ol	74 (39%), 292 (15%) M-32, 324 (1%) M+
10-Nonadecen-2-one	58, 280 (M+), 262 (M-18)
N,N-Dimethylhydrazone of 10-nonadecen-2-one	100 (100%), 322 (16%) M+, 278 (12%) M-44
Dithiomethyl ether derivative of 10-nonadecen-2-one	173 (46%), 201 (100%) 374 (12%) M+
N,N-Dimethylhydrazone of 10,11-di(thiomethyl) nonadecen-2-one	100 (100%), 173 (16%), 243 (11%), 416 (16%) M+
Methyl ester of Δ^x-octadecenoic acid	74 (24%), 264 (13%) M-32 296 (2%) M+
Methyl ester of octadecanoic acid	74 (100%), 267 (3%) M-31 298 (8%) M+
Δ^x-Nonadecenal	262 (M-18)
N,N-Dimethylhydrazone of Δ^x-nonadecenal	86 (100%), 322 (14%) M+, 278 (10%) M-44
Δ^x-Eicosenal	82, 96, 294 (M+), 276 (M-18)
N,N-Dimethylhydrazone of Δ^x-eicosenal	86 (100%), 336 (25%) M+, 292 (5%) M-44

tion of this compound with dimethylhydrazine (DMH) to make the corresponding dimethylhydrazone yielded major cleavages by mass spectrometry at (*m/z*) 173 and 243, thus identifying the double-bond position as being at carbon 10.

Treatment of the polar fraction of the male gland extract with dimethylhydrazine resulted in the formation of *N,N*-dimethylhydrazones of 10-eicosenal, and possibly a small amount of Δ^x-nonadecenal. DMH methylated the fatty acids present (McDaniel and Howard, 1985) in this fraction. The acids identified were Δ^x-octadecenoic acid and octadecanoic acid. The mass spectral data of these compounds and their derivatives are presented in Table 2.

DISCUSSION

The mandibular gland secretions of male *P. triangulum* are strikingly different from those of its North American counterparts. As illustrated in Table 3, the secretion of *P. triangulum* differs in the chemical functionalities of compounds present, in numbers of compounds present, and in the sizes of the compounds. About 90% of the *P. triangulum* secretion is the C_{20} alcohol (Z)-11-eicosen-1-ol and 5% is the unsaturated C_{19} ketone 10-nonadecen-2-one, both compounds with functionalities, or combinations of functionalities, entirely absent in the glandular secretions of *P. basilaris* and *P. bicinctus*. The *P. triangulum* secretion thus contains primarily one compound and a total blend of only six compounds. All except the two fatty acids have carbon skeletons of either 19 or 20 carbons. These two fatty acids represent about 3.7% of the secretion mixture. The glandular secretions of *P. basilaris* and P. bicinctus, by contrast, possess secretions rich with saturated ketones, fatty acids, and ethyl esters of the fatty acids. Eight compounds of these types comprise 96–98% of the total secretion of *P. basilaris*, and 13 of these compounds comprise 93–99% of the total secretion of *P. bicinctus*. Combined with fatty acid components, the North American species possess complex mixtures of 10 and 14 compounds, respectively, none of which has a carbon skeleton greater than 18 carbons. This suggests that the nonacid portion of *P. basilaris* and *P. bicinctus* secretions would exhibit greater volatility than the components of *P. triangulum*. Aldehydes appear to be minor components in the secretions of all three *Philanthus* species.

(Z)-11-Eicosen-1-ol is previously unreported from a wasp species. It has been identified as a secretion in *Apis mellifera*, the prey of *P. triangulum*, where it is found in the sting chamber of workers and functions as an alarm pheromone (Pickett et al., 1982) and possibly as a general attractant for foragers (Free et al., 1982). We could detect no alkylpyrazines in *P. triangulum*. Borg-Karlson and Tengö (1980) reported up to 200 ng of pyrazines in this species, but since they were specifically looking for pyrazines and terminated their GC temperature program at 150°C, they did not observe the higher-molecular-weight com-

TABLE 3. COMPARISON OF MALE MANDIBULAR GLAND COMPOUNDS AMONG *Philanthus* SPECIES

Chemical classifications and compounds	Compositions (%) of mandibular gland		
	P. basilaris[a]	*P. bicinctus*[a]	*P. triangulum*
Alcohols			
(Z)-11-Eicosen-1-ol			89.5
Saturated ketones			
2-Tridecanone	0.4	0–0.1	
2-Pentadecanone	53–54	24–53	
2-Heptadecanone	0.2–0.4	0–3	
Unsaturated ketones			
10-Nonadecen-2-one			5.2
Aldehydes			
Δ^x-Heptadecenal[b]	0.1–0.3		
Δ^x-Octadecenal	2–3		
Δ^x-Nonadecenal		0.2–7	1.7
Δ^x-Eicosenal			trace
Fatty acids			
Hexadecanoic acid	5–7	1–15	
Δ^x-Hexadecenoic acid	26–27	16–29	
Octadecanoic acid	3	1–7	
Δ^x-Octadecenoic acid + $\Delta^{x,y}$-Octadecadienoic acid	7–10	3–28	3.7
Esters			
Ethyl hexadecanoate		1–11	
Ethyl Δ^x-hexadecenoate		10–33	
Ethyl octadecanoate		1–9	
Ethyl Δ^x-octadecenoate + ethyl $\Delta^{x,y}$-octadecadienoate		3–14	

[a] Data from Schmidt et al. (1985).
[b] Symbols Δ^x and $\Delta^{x,y}$ indicate positions of double bonds are unknown.

pounds reported here. (Z)-11-Eicosen-1-ol and the minor components reported here are, however, more likely to be the true male pheromones, since they are present in microgram rather than nanogram quantities and are present only in males.

The chemistry of the male cephalic secretions in *Philanthus* provides a tool for investigating systematics and evolution within the genus. Based on morphological data the North American species, *P. basilaris* and *P. bicinctus* have been variously listed in the genus (or subgenus) *Ocloclетes*, or afforded placement at least in the *zebratus* species group (Evans and O'Neill, 1988), whereas *P. triangulum* is not closely related. Our chemical data (plus unpublished data

from several other North American species not in the *zebratus* group) indicate that North American species are clearly distinct from *P. triangulum*. Males of all of these species defend scent-marked territories; however, males of several North American *Philanthus* species often form territories at the same location at the same time (Evans and O'Neill, 1988). Thus, these species have an apparent problem of species isolation. *P. triangulum*, on the other hand, has few, if any, interactions with sympatric congenerics in western Europe and, therefore does not need species-isolating mechanisms. Without selection pressure from congenerics, the role of the male secretion of *P. triangulum* can be that of a simple attractant pheromone that contains only one major component plus a few minor components.

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ALLELOPATHIC POTENTIAL OF COMPOUNDS ISOLATED FROM *Ipomoea tricolor* CAV. (CONVOLVULACEAE)[1]

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Abstract—The allelopathic potential of *I. tricolor*, used in traditional agriculture as a weed controller, has been demonstrated by measuring the inhibitory activity of aqueous lixiviates and organic extracts of the plant material on seedling growth of *Amaranthus leucocarpus* and *Echinochloa crusgalli*. Bioactivity-guided fractionation of the most active organic extract led to the isolation of the allelopathic principles, which turned out to be a mixture of glycosides, having jalapinolic acid as the aglycone portion glycosidically linked in the 11 position to an oligosaccharide composed of glucose, rhamnose, and fucose, which also combines with the carboxyl group of the aglycone to form a macrocyclic ester.

Key Words—Allelopathy, *Ipomoea tricolor*, Convolvulaceae, traditional agroecosystems, natural weed controller, glycosidic resins.

INTRODUCTION

The genus *Ipomoea* (Convolvulaceae) comprises about 600 species, and its richness of secondary metabolites with biological activity is perhaps its most remarkable characteristic. In Mexico, the seeds of *Ipomoea tricolor* (tlitlitzin, badoh negro or don Diego de dia) as well as those of the related species *Turbina corymbosa* (L.) Raf. (ololiuqui or snake plant), are used as hallucinogenics in

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magic rituals. The biological activity is due to their content of ergot type alkaloids (Tyler et al., 1988). Other species of *Ipomoea* are widely used all over the world as powerful cathartics (Shellard, 1961). This activity is related to the presence in those plants of glycosidic resins, which are the most common constituents in the genus (Noda et al., 1987, among others). It has also been demonstrated that these glycosides contain antimicrobial and antitumoral activity (Bhakuni et al., 1969; Sarin et al., 1973; Bieber et al., 1986).

In the tropical zones of Mexico, peasants who use a traditional management of resources in agroecosystems know how to use some weeds to control the growth of others (Ramos et al., 1983; Anaya et al., 1987a,b). In the state of Tabasco, as Chacon and Gliessman reported (1982), they are able to distinguish good ("buen monte") from bad weeds ("mal monte") and promote the cultivation of the former and elimination of the latter. The cultivation of some semidomesticated legume species, especially *Stizolobium pruriens*, has rapidly increased to eliminate weeds. Some *Ipomoea* species are also used for this purpose. In the sugar cane fields of the state of Morelos, Mexico, *Ipomoea tricolor* is commonly grown extensively as a cover crop, especially from August to October (Anaya, 1989). Peasants practice this before sowing and during the fallow period (Figure 1). They know that this plant will eliminate all other weeds in two or three months. After this time, they cut the plant and incorporate it into the soil as green manure.

Previous allelopathic studies on the related species *I. aquatica* and *I. batatas* showed that the terpenoid and phenolic components were responsible for the observed inhibitory activity of these plants (Singhvi and Sharma, 1984; Howard and Peterson, 1986).



FIG. 1. A dense population of *Ipomoea tricolor* growing in a sugar cane field in Morelos, Mexico.

METHODS AND MATERIALS

Plant Material. Whole plants of *Ipomoea tricolor* (2 kg) were collected in Cañon de Lobos, Morelos, in February 1988. A voucher specimen (Anaya, No. 88-I) was deposited at the Herbario Nacional, Instituto de Biología, UNAM.

General Bioassay Procedures. The allelopathic potential of *I. tricolor* was tested on seeds of *Amaranthus leucocarpus* and *Echinochloa crusgalli*. The bioassays were made in Petri dishes. Each aqueous lixiviate (10 ml), obtained from fresh and air-dried plant material (15% and 1%, respectively), was mixed separately with 1.5% agar (10 ml). Distilled water mixed with agar was used as control. The osmotic pressure of all lixiviates was measured in a freezing-point osmometer. A complete random block design with four repetitions for each target species was used. In each case 40 seeds were tested. The Petri dishes were kept at 27°C in the dark. After 24 hr for *Amaranthus* and 48 hr for *Echinochloa*, the radicle length was measured. The results were processed by ANOVA.

The inhibitory activity was also determined for different organic extracts, column chromatography fractions, and the glycosidic resin fraction. In these cases, the materials were dissolved in a suitable organic solvent and then poured on a filter paper disk. After evaporation, distilled water was added and the bioassay was carried out under the same general conditions.

Extraction and Fractionation Procedures. For preliminary biological evaluations, crude extracts of air-dried whole plant were obtained by macerating a 100-g portion of the material with *n*-hexane, chloroform, ethyl acetate, and methanol for 48 hr. After evaporation of the solvent, the respective residues at different concentrations were assayed. To evaluate the bioactivity of the alkaloidal fraction, a 100-g portion of the plant material previously made basic was extracted with chloroform. The resulting extract was subjected to conventional acid–base partitioning to yield 50 mg of an alkaloidal crude residue.

Since the highest inhibitory activity was observed in the ethyl acetate extract, 1.5 kg of plant material, previously defatted with *n*-hexane, were macerated at room temperature with 7.5 liter of EtOAc. After filtration and evaporation of the solvent, the residual material was dissolved in $CHCl_3$ and decolorized with activated charcoal followed by vacuum drying to obtain 74 g of an oily residue. This extract was column chromatographed on 726 g of silica gel (60 Merck, 70–230 mesh); mixtures of *n*-hexane–$CHCl_3$ and $CHCl_3$—MeOH were used as eluents. A total of 873 fractions (100 ml each) was obtained. Combined fractions 760–771 (9 g) eluted with $CHCl_3$—MeOH (4 : 1) displayed the inhibitory activity. Further column chromatography on silica gel of 5 g of this material, using EtOAc as eluent, resulted in the isolation of 1.6 g (0.192% of dry wt) of a white crystalline glycosidic mixture, mp 109–111°C, as the only active component.

Alkaline Hydrolysis of Active Material. The active component (400 mg) was stirred in a solution of 400 mg of NaOH in 8 ml of H_2O. After 4 hr at room temperature, the solution was acidified with 4 N H_2SO_4 and extracted with ether. The residue obtained after elimination of the solvent was methylated with ethereal diazomethane and subjected to GC-MS. The acidic solution was refluxed for 8 hr. From this solution, there precipitated spontaneously 66 mg of a yellowish powder, mp 57–59°C. After removal of the solid material by filtration, the acid solution was extracted with $CHCl_3$; evaporation of the solvent yielded additional amounts (5 mg) of the solid compound. The precipitated solid compound (60 mg) was treated with ethereal diazomethane to yield 50 mg of methyl jalapinolate, mp 40–43°C; $[\alpha]_D$ +0.9° (c 0.7, $CHCl_3$); EI-MS *m/z* (rel. abundance) 286 (M^+; 2), 255 (3), 229 (15), 215 (25), 200 (15), 183 (65), 157 (20), 143 (35), 101 (60), 87 (90), 74 (40), 59 (20), 55 (100); [^{1}H]NMR (δ, $CDCl_3$): 0.87 (3H, t, J = 7 Hz, H-16), 2.25 (2H, t, J = 7 Hz, H-2), 3.46 (1H, m, H-11), 3.56 (3H, s, $-OCH_3$); IR absorption (neat) (cm^{-1}): 3350, 2900, 2850, 1745.

The aqueous layer left after both hydrolyses was neutralized by filtering through an anion exchange column (Ionenaustauscher II, Merck) and then evaporated to dryness. The residue was taken up in MeOH. The presence of glucose, fucose, and rhamnose was made evident by TLC on silica gel impregnated with NaH_2PO_4 0.2 M or with H_3BO_3 0.036 M and using acetone–H_2O (9:1) or *n*-BuOH—H_2O—HOAc (4:1:1) as elution systems (Ghebregzabher et al., 1976).

Identification. Melting points are uncorrected; IR spectra were recorded on a Perkin-Elmer 599-B instrument on KBr disks or neat; [^{1}H]NMR spectra were registered on a varian FT-80 spectrometer in $CDCl_3$ or $CDCl_3$—DMSO—d_6 solutions with TMS as internal standard. For GC-MS, a methylsilicone MB capillary column (25 × 0.32 mm) was programmed from 180 to 260°C at 8°C/min; and the mass spectral (EI-MS) measurements were carried out on a Hewlett Packard 5985 machine at 70 eV at the Instituto de Quimica, UNAM. Optical rotations were measured on a Perkin-Elmer model 141 polarimeter.

RESULTS AND DISCUSSION

Preliminary Bioassay. The results obtained in the bioassays with lixiviates of fresh and dry *Ipomoea tricolor* are summarized in Table 1. There was a strong and significant growth inhibition on the test species by all the treatments. Dry *I. tricolor* lixiviates caused less inhibition of *Echinochloa* than fresh lixiviates.

The preliminary results with the organic extracts are reported in Table 2. The most inhibitory effects were induced by ethyl acetate and chloroform

TABLE 1. EFFECT OF AQUEOUS LIXIVIATES OF *I. tricolor* ON RADICLE GROWTH OF *Amaranthus* AND *Echinochloa*

Aqueous lixiviates of *I. tricolor*	Radicle growth (cm)		Growth inhibition (%)	
	Amaranthus	*Echinochloa*	*Amaranthus*	*Echinochloa*
Control	31.28 ± 1.65	19.12 ± 1.00		
Lixiviate fresh material (15%)	10.58 ± 0.47	8.62 ± 0.94	66.19[a]	54.9[a]
Control	13.95 ± 0.51	17.98 ± 0.33		
Lixiviate dry material (1%)	5.72 ± 0.21	8.35 ± 0.58	57.12[a]	53.55[a]

[a] $P < 0.01$.

extracts. The alkaloidal fraction was also inhibitory; the observed effect was 66% inhibition of *Amaranthus* and 45% of *Echinochloa* (Table 3).

Fractionation of Ethyl Acetate Extract and Biological Activity. In order to separate the active principles, the ethyl acetate extract was fractionated by column chromatography; the resin glycosidic fractions were the most inhibitory to seedling growth (Table 4). Further chromatographic analysis of this active portion led to the isolation of a crystalline glycosidic mixture, which turned out to cause the allelopathic activity of the plant (Figure 2).

TABLE 2. EFFECT OF ORGANIC EXTRACTS OF *I. tricolor* ON RADICLE GROWTH OF *Amaranthus* AND *Echinochloa*

Organic extracts of *I. tricolor*	Radicle growth (cm)		Growth inhibition (%)	
	Amaranthus	*Echinochloa*	*Amaranthus*	*Echinochloa*
Control	12.80 ± 0.41	22.85 ± 0.89		
Hexane extract (100 ppm)	11.72 ± 0.85	22.60 ± 1.44	8.40	1.09
Control	14.40 ± 0.30	20.33 ± 1.13		
Chloroform extract (100 ppm)	8.73 ± 0.47	14.60 ± 1.30	39.35[a]	28.20[a]
Control	14.77 ± 0.77	24.53 ± 1.33		
Ethyl acetate extract (100 ppm)	10.77 ± 0.57	15.97 ± 1.48	27.09[a]	34.92[a]
Control	17.48 ± 0.27	32.48 ± 1.84		
Methanol extract (100 ppm)	16.50 ± 0.32	29.98 ± 0.66	5.58	7.70

[a] $P < 0.01$.

TABLE 3. EFFECT OF ALKALOIDAL CRUDE AND ETHYL ACETATE EXTRACTS OF *I. tricolor* ON RADICLE GROWTH OF *Amaranthus* AND *Echinochloa*

Organic extracts of *I. tricolor*	Radicle growth (cm)		Growth inhibition (%)	
	Amaranthus	*Echinochloa*	*Amaranthus*	*Echinochloa*
Control	12.73 ± 0.26	25.27 ± 0.98		
Alkaloidal crude (100 ppm)	4.30 ± 0.40	14.03 ± 0.87	66.23[a]	44.46[a]
Control	18.15 ± 0.57	26.00 ± 0.44		
Ethyl acetate decolorized (100 ppm)	3.82 ± 0.63	3.82 ± 0.95	78.92[a]	86.25[a]

[a] $P < 0.01$.

The crystalline material gave a positive reaction with Molisch reagent and did not react with ethereal diazomethane. Its IR spectrum displayed characteristic absorptions for hydroxyl (3450, 1070 cm^{-1}) and lactone (1750 cm^{-1}) functionalities. Alkaline hydrolysis with NaOH liberated a mixture of 2-methylbutyric and 3-hydroxy-2-methylbutyric acids, which were identified as their methyl esters by GC-MS and comparison with authentic samples. No free sugar was detected after saponification. Subsequent acid hydrolysis yielded exclusively jalapinolic acid (11-hydroxyhexadecanoic acid) as the aglycone moiety. This unit was characterized as its methyl ester, for which all the physical and spectroscopic parameters were identical with those previously described (Noda et al., 1988a; Bieber et al., 1986, among others). In the aqueous layers

TABLE 4. EFFECT OF CHROMATOGRAPHIC FRACTIONS OF ETHYL ACETATE EXTRACT OF *I. tricolor* ON RADICLE GROWTH OF *Amaranthus* AND *Echinochloa*

Fractions	Radicle growth (cm)		Growth inhibition (%)	
	Amaranthus	*Echinochloa*	*Amaranthus*	*Echinochloa*
Control	15.80 ± 0.25	25.30 ± 0.84		
670–684 (100 ppm)	3.50 ± 1.44	11.22 ± 1.23	81.01[a]	55.63[a]
Control	17.52 ± 0.41	28.70 ± 1.59		
685–695 (100 ppm)	14.02 ± 0.30	11.28 ± 0.46	19.97	60.71[a]
Control	17.68 ± 0.73	25.75 ± 0.74		
760–771 (100 ppm)	6.40 ± 0.18	10.50 ± 0.42	63.79[a]	68.93[a]
Control	15.80 ± 0.25	25.30 ± 0.84		
772–850 (100 ppm)	9.50 ± 0.99	16.05 ± 1.77	39.87[a]	36.56[a]

[a] $P < 0.01$.

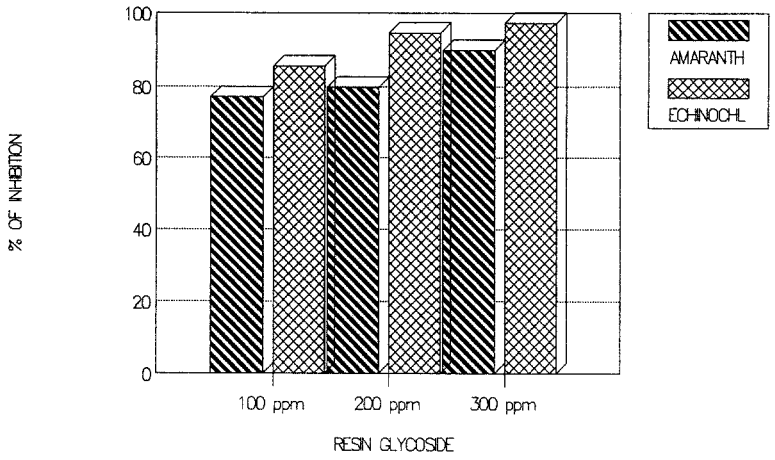


FIG. 2. Percentage of inhibition of the radicle growth of *Amaranthus* and *Echinochloa* caused by the resin glycoside mixture obtained from *Ipomoea tricolor* at three concentrations (all data are statistically significant at the 1% level).

of the acidic hydrolysates, glucose, fucose, and rhamnose were detected by TLC and PC as the only sugar components.

These results indicate that the active compounds are a typical resin glycoside mixture like those previously found in other species of the genus (e.g., Wagner et al., 1978; Harrison et al., 1985; Bieber et al., 1986; Noda et al., 1987, 1988b,c; Ono et al., 1989). All the glycosides of the mixture contain jalapinolic acid as the aglycone portion glycosidically linked in the C-11 position to the oligosaccharide, which in turn combines with the carboxyl group to form a macrolactone. Finally, as previously described, the small fatty acids must be esterifying the sugars at different positions.

The practical use of *Ipomoea tricolor* as a weed controller in traditional agroecosystems, as well as the allelopathic activity of resin glycosides, offer promise as natural herbicides. Currently, further studies are in progress in order to separate and characterize the individual components of this active mixture.

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(3Z,6Z,9Z)-NONADECATRIENE AND ENANTIOMERS OF (3Z,9Z)-*cis*-6,7-EPOXY-NONADECADIENE AS SEX ATTRACTANTS FOR TWO GEOMETRID AND ONE NOCTUID MOTH SPECIES[1]

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Abstract—Sex attractants for the geometrid moths *Eufidonia convergaria* and *Caripeta angustiorata*, and the noctuid moth *Rivula propinqualis* have been elucidated during field screening of a series of (3Z,6Z,9Z)-triene hydrocarbons (C_{17-22}), and the racemic and enantiomerically enriched monoepoxydienes derived from those hydrocarbons. Biologically active compounds were identified by a combination of field testing of synthetic standards, electroantennography, and coupled gas chromatography–electroantennogram detection. *E. convergaria* males were optimally attracted by a 1:1 blend of (3Z,9Z)-(6*S*,7*R*)-epoxy-nonadecadiene (3Z,9Z-6*S*,7*R*-epoxy-19:H); other abbreviations follow the same system) with (3Z,6Z,9Z)-nonadecatriene (3Z,6Z,9Z-19:H). The 6*R*,7*S* enantiomer of the epoxide had no apparent biological activity, either as an attractant or as a behavioral antagonist. Male moths also were attracted to blends of the C_{18} and C_{20} homologs of the triene and the epoxide. 3Z,6Z,9Z-19:H and 3Z,6Z-*cis*-6,7-epoxy-19:H were identified in *E. convergaria* female pheromone gland extracts. Males of the geometrid moth species *C. angustiorata* were attracted by a 1:1 blend of 3Z,6Z,9Z-19:H and enantiomerically enriched 3Z,9Z-6*R*,7*S*-epoxy-19:H. Males of the noctuid moth *R. propinqualis* were attracted by an approximately 10:1 blend of 3Z,6Z,9Z-19:H and enantiomerically enriched 3Z,9Z-6*S*,7*R*-epoxy-19:H. The components were synergistic, with neither being attractive alone. The blend ratio was quite specific, as the attractiveness of blends decreased sharply on either side of the optimum ratio.

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INTRODUCTION

To date, there have been relatively few sex pheromones or sex attractants identified for moths in the family Geometridae. From the reports so far, the pheromones or attractants usually consist of all-*cis* diene, triene, or tetraene hydrocarbons of chain lengths C_{17-21}, or monoepoxides derived therefrom, alone or in combination (compilation to 1986, Arn et al., 1986; McDonough et al., 1986; Becker et al., 1987; Millar et al., 1987; Szöcs et al., 1987; Millar et al., 1989a; 1990a,b). As a continuation of our studies on the synthesis and field screening of 3Z,6Z,9Z-triene hydrocarbons and the epoxides derived therefrom, and the elucidation of these compounds as sex attractants and sex pheromones, we report here sex attractant blends for the geometrid moths *Eufidonia convergaria* Walker and *Caripeta angustiorata* Walker and for the noctuid moth *Rivula propinqualis* Guenée. We also report that the two compounds in the sex attractant blend for *Eufidonia convergaria* have been identified in extracts of sex pheromone glands from *E. convergaria* female moths.

METHODS AND MATERIALS

Insects and Electroantennography. Male moths for electroantennogram (EAG) and coupled gas chromatography–electroantennogram detection (GC-EAD) studies were captured in light traps, in sweep nets, or in traps baited with synthetic sex attractants. Female moths of unknown mating status were taken in light traps or in sweep nets. Male antennal responses to synthetic compounds were measured by EAG (Chisholm et al., 1975), using 1-μg amounts of compounds applied to filter paper disks, and by the GC-EAD method of Arn et al. (1975). Synthetic compounds tested in EAG and/or GC-EAD studies generally included C_{17-22} chain lengths of the following types of compounds: 6Z,9Z-, 3Z,6Z,9Z-, 1,3Z,6Z,9Z-, 3Z,6Z,9Z,11Z-, and 3Z,6Z,9Z,11*E*-unsaturated hydrocarbons; combined mixtures of the monoepoxide regioisomers from nonselective oxidation of the diene or triene hydrocarbons above (combined monoepoxides, CME); racemic and chiral forms of 3Z,6Z-*cis*-9,10-, 3Z,9Z-*cis*-6,7-, and 6Z,9Z-*cis*-3,4-epoxydienes; racemic and highly enantiomerically enriched forms of 6Z-*cis*-9,10- and 9Z-*cis*-6,7-epoxymonoenes. Other compounds were tested as they became available through synthesis and as was deemed appropriate from preliminary data from field captures.

Extracts of sex pheromone glands from field-collected female moths were prepared by a 20-min pentane extraction of the terminal three to four abdominal segments. The extracts were concentrated under a stream of nitrogen, internal standards (*n*-heptadecane and *n*-tetracosane) were added, and the extract was submitted to GC-EAD as previously described (Millar et al., 1987). The signals from the flame ionization detector and the insect antenna were recorded simultaneously with a matched pair of Hewlett-Packard 3392A integrating chart recorders, which recorded the retention times of each FID and EAD peak directly on the recording charts.

Coupled gas chromatography–mass spectrometry (GC-MS) analyses were carried out in selected ion monitoring (SIM) mode, using chemical ionization (isobutane), with a Finnigan 4000E instrument interfaced to an Incos 2300 data system. GC-MS analysis of an *E. convergaria* female pheromone gland extract was conducted as follows. Full-scan CI mass spectra of synthetic standards were obtained to select characteristic ions. A dilute pentane solution of the standards (3Z,6Z,9Z-19 : H and 3Z,9Z-*cis*-6,7-epoxy-19 : H, with octadecane and tetracosane internal standards; 400 pg of each compound injected) was then run in SIM mode, followed by a blank run. Finally, the concentrated pentane extract of the pheromone glands from four field-collected female moths of unknown mating status was analyzed in SIM mode (pentane solution of octadecane and tetracosane internal standards added prior to injection).

Insect Trapping. Field experiments were carried out in a mixed forest area approximately 100 km northeast of Saskatoon. Pherotech (Vancouver, British Columbia) wing-traps, similar in design to Pherocon 1CP traps (Scentry Inc., Buckeye, Arizona) were used throughout and were baited with red rubber septa containing the synthetic lure blends. These lure blends (usually 500 μg of test compound total weight) were loaded onto septa as hexane solutions. Baits were stabilized by the addition of several drops of a dilute acetone solution of butylated hydroxytoluene.

Traps were hung from branches 10–15 m apart, at a height of ca 1.5 m and were set out in a randomized block design. Moth captures were recorded twice weekly, and summed trap captures were transformed $[(X + 1/2)^{1/2}]$ and subjected to analysis of variance. The variances of the transformed data were tested for homogeneity with Bartlett's test (Sokal and Rohlf, 1981). Treatments with zero trap captures overall were not included in the ANOVA, as ANOVA requires that there be some variance within a treatment. Differences between treatment means were tested by Duncan's (1955) multiple-range test.

Field survey traps in 1984 were baited with the following C_{18-22} compounds, alone or as binary mixtures at ratios of 10 : 1 and 1 : 10: 3Z,6Z,9Z-trienes, 6Z,9Z-dienes, combined monoepoxides mixtures of those dienes and trienes (CME-X : H), racemic *cis*-3,4-, *cis*-6,7-, and *cis*-9,10-epoxydienes, and 3Z,6Z,9Z,11*E*-tetraenes.

Field survey traps in 1985 were baited with the following compounds, generally in chain lengths of C_{16-22}, alone or as binary mixtures at ratios of 10:1 and 1:10: 3*Z*,6*Z*,9*Z*,12*Z*-, 3*Z*,6*Z*,9*Z*,13*E*-, 3*Z*,6*Z*,9*Z*,13*Z*-, 3*Z*,6*Z*,9*Z*,14*Z*-, 3*Z*,6*Z*,9*Z*,14*E*-tetraenes; 3*Z*,6*Z*,9*Z*-trienes; enantiomers of *cis*-6,7- and *cis*-9,10-epoxydienes. Some ternary mixtures of compounds were also fielded.

Field survey traps in 1986 were baited with the following compounds, generally in chain lengths of C_{16-21}, alone or as binary mixtures at ratios of 10:1 and 1:10: 3*Z*,6*Z*,9*Z*-trienes; 1,3*Z*,6*Z*,9*Z*-tetraenes; enantiomers of 6*Z*,9*Z*-*cis*-3,4-epoxydienes; 6*Z*,9*Z*-dienes; enantiomers of 6*Z*-*cis*-9,10- and 9*Z*-*cis*-6,7-epoxymonoenes; 6*Z*,9*Z*-dien-3-ols and 6*Z*,9*Z*-dien-3-ones. Some ternary baits were also set out.

Synthetic Chemicals. The syntheses of the compounds used in the field surveys and EAG or GC-EAD studies described in this paper have been published elsewhere, as follows: 6*Z*,9*Z*- and 3*Z*,6*Z*,9*Z*-unsaturated hydrocarbons and the corresponding *cis*-3,4-, *cis*-6,7-, and *cis*-9,10-monoepoxides (Underhill et al., 1983; Wong et al., 1985; Millar et al., 1987); enantiomers of *cis*-9,10-epoxydienes (Wong et al., 1985); enantiomers of *cis*-6,7-epoxydienes (Millar and Underhill, 1986); enantiomers of *cis*-3,4-epoxydienes (Millar et al., 1989b).

With regard to the specific experiments described in this report, the 3*Z*,6*Z*,9*Z*-19:H was >99% chemically and isomerically pure by capillary gas chromatography (DB-5 and DB-1701 columns). The enantiomers of 3*Z*,9*Z*-*cis*-6,7-epoxy-19:H were >99% chemically pure by capillary GC, with enantiomeric excesses of 88.3% (6*R*,7*S* enantiomer) (Millar and Underhill, 1986).

RESULTS

Larvae of the geometrid moth *Eufidonia convergaria* feed on pine, but infestation levels in central Saskatchewan are never high enough to cause serious damage. Male *Eufidonia convergaria* moths were first caught in 1984, in traps containing *cis*-6,7-epoxydienes of chain lengths C_{18-20}. In 1985, large numbers of these moths were caught in field surveys, primarily in replicated traps ($N = 2$) baited with a mixture of a triene hydrocarbon (C_{18-20}) and either enantiomer of a 3*Z*,9*Z*-*cis*-6,7-epoxydiene, also of chain length C_{18-20}. A variety of blend ratios were attractive.

GC-EAD of a standards mixture and of a pheromone gland extract from a field-collected female were crucial in elucidating the optimum blend of attractant components (Figure 1). A male antenna responded to a number of components in the standards mixture, including 3*Z*,6*Z*,9*Z*-18:H, 3*Z*,6*Z*,9*Z*-19:H, and the *cis*-3,4- and *cis*-6,7-epoxydienes of chain lengths 18–20 carbons (Figure 1, top). However, the extract of the pheromone gland from a single field-

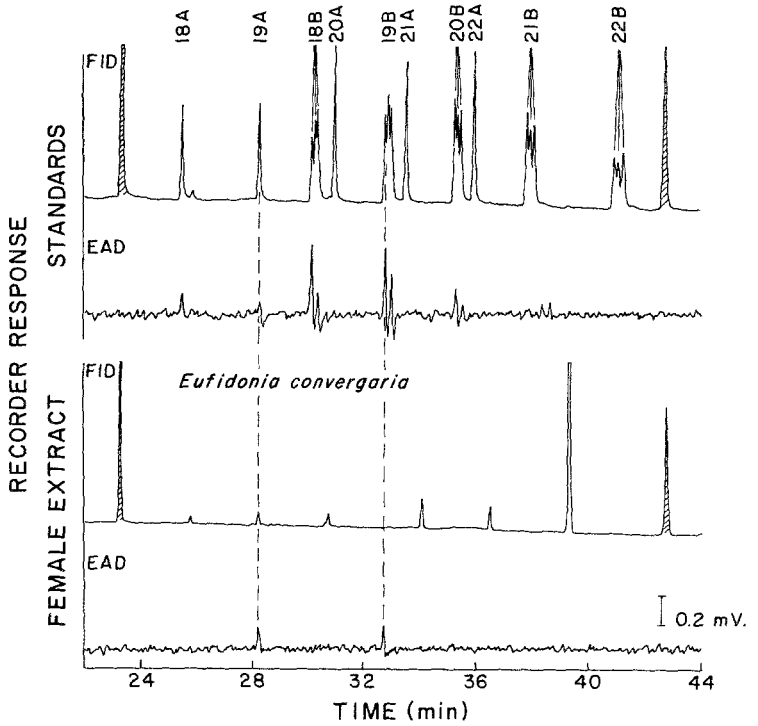


FIG. 1. Simultaneously recorded flame ionization detector (FID) and electroantennographic detector (EAD) traces using antennae of male *Eufidonia convergaria*. Upper pair are in response to a mixture of C_{18-22} 3Z,6Z,9Z-triene hydrocarbons (designated by the letter A and preceded by the carbon chain length) and their monoepoxydiene hydrocarbon analogs (designated by the letter B); the latter eluted from the 30-m DB-5 column in the order 6,7-, 3,4-, and 9,10-monoepoxydiene. Internal standards: 17:H and 24:H (shaded). Lower pair are in response to an abdominal tip extract of a conspecific female.

collected female of unknown mating status elicited only two responses from the male antenna, at retention times corresponding to 3Z,6Z,9Z-19:H and 3Z,9Z-*cis*-6,7-epoxy-19:H (Figure 1, bottom). It should be noted that the amount of the antennal stimulatory compound in the pheromone gland extract at the retention time of 3Z,9Z-*cis*-6,7-epoxy-19:H was below the detection threshold of the flame ionization detector (Figure 1, bottom), illustrating the sensitivity of the antenna.

The possible presence of 3Z,6Z,9Z-19:H and 3Z,9Z-*cis*-6,7-epoxy-19:H in pheromone gland extracts was corroborated by GC-MS of a pheromone gland extract from four field-collected females of unknown mating status, using isobutane chemical ionization (CI) in selected ion (SIM) mode. Excellent retention

time and good ion ratio matches were obtained for 3*Z*,6*Z*,9*Z*-19:H [standard, *m/z* 319 (53%; M + 57), 263 (65%; M + 1), 261 (100%; M − 1), 221 (23%; M + 1 − 42), 206 (28%; M + 1 − 57); unknown in extract, *m/z* 319 (55%), 263 (68%), 261 (100%), 221 (14%), 206 (42%)] and for 3*Z*,9*Z*-*cis*-6,7-epoxy-19:H [standard, *m/z* 279 (100%; M + 1), 263 (6%; M − 15), 261 (59%; M + 1 − 18), 209 (13%; M + 1 − 70); unknown in extract, *m/z* 279 (100%), 263 (12%), 261 (56%), 209 (8%)].

Specific field experiments confirmed that blends of 3*Z*,6*Z*,9*Z*-19:H and 3*Z*,9*Z*-6*S*,7*R*-epoxy-19:H were highly attractive (Table 1), with the optimum blend ratio being approximately 1:1.

The data also indicated that considerably different blends of 3*Z*,6*Z*,9*Z*-19:H with the other epoxide enantiomer, 3*Z*,9*Z*-6*R*,7*S*-epoxy-19:H, were attractive. However, it seems likely that this is an artifact caused by the enantiomeric impurity in the synthetic 6*R*,7*S* enantiomer. The synthetic route used to make the chiral epoxides gave an enantiomeric excess of approximately 88% (i.e., a 94:6 mixture of the enantiomers) (Millar and Underhill, 1986). Thus, this 6% optical impurity, when combined with an roughly equal amount of 3*Z*,6*Z*,9*Z*-19:H, would approximate the optimally attractive blend, inducing some attraction, albeit considerably less due to the lower overall dose of attrac-

TABLE 1. CAPTURES OF *Eufidonia convergaria* IN TRAPS BAITED WITH BLENDS OF 3*Z*,6*Z*,9*Z*-19:H WITH ENANTIOMERICALLY ENRICHED 3*Z*,9*Z*-*cis*-6,7-EPOXY-19:H[a]

Lure composition (μg)	Total males captured[b]
3*Z*,9*Z*-6*R*,7*S*-epoxy-19:H (500)	7de
3*Z*,9*Z*-6*R*,7*S*-epoxy-19:H (475) + 3*Z*,6*Z*-9*Z*-19:H (25)	34bcd
3*Z*,9*Z*-6*R*,7*S*-epoxy-19:H (400) + 3*Z*,6*Z*-9*Z*-19:H (100)	46bc
3*Z*,9*Z*-6*R*,7*S*-epoxy-19:H (250) + 3*Z*,6*Z*-9*Z*-19:H (250)	11cde
3*Z*,9*Z*-6*R*,7*S*-epoxy-19:H (100) + 3*Z*,6*Z*-9*Z*-19:H (400)	6de
3*Z*,9*Z*-6*R*,7*S*-epoxy-19:H (25) + 3*Z*,6*Z*-9*Z*-19:H (475)	0
3*Z*,9*Z*-6*S*,7*R*-epoxy-19:H (500)	0
3*Z*,9*Z*-6*S*,7*R*-epoxy-19:H (475) + 3*Z*,6*Z*-9*Z*-19:H (25)	21cde
3*Z*,9*Z*-6*S*,7*R*-epoxy-19:H (400) + 3*Z*,6*Z*-9*Z*-19:H (100)	69ab
3*Z*,9*Z*-6*S*,7*R*-epoxy-19:H (250) + 3*Z*,6*Z*-9*Z*-19:H (250)	133a
3*Z*,9*Z*-6*S*,7*R*-epoxy-19:H (100) + 3*Z*,6*Z*-9*Z*-19:H (400)	35bcd
3*Z*,9*Z*-6*S*,7*R*-epoxy-19:H (25) + 3*Z*,6*Z*-9*Z*-19:H (475)	2e
3*Z*,6*Z*-9*Z*-19:H (500)	0

[a] Enantiomeric excess of 3*Z*,9*Z*-6*R*-7*S*-epoxy-19:H, 88.3%; 3*Z*,9*Z*-6*S*,7*R*-epoxy-19:H, 88.2% ee.
[b] Replicated traps ($N = 3$) were set out June 14–28, 1985. Values followed by the same letter are not significantly different ($P > 0.05$).

tive materials. This also suggested that the 6*R*,7*S* enantiomer was not behaviorally antagonistic, even at relatively large doses.

A further experiment conducted in 1986 confirmed these results. Four baits containing the optimum 1 : 1 blend of 3*Z*,6*Z*,9*Z*-19 : H with 3*Z*,9*Z*-6*S*,7*R*-epoxy-19 : H were spiked with small to large amounts of 3*Z*,9*Z*-6*R*,7*S*-epoxy-19 : H (amounts added were 0–100% of the amount of attractants). There were no significant differences in the attractiveness of baits (data not shown; total of 425 male moths caught in all traps), confirming that the 6*R*,7*S* enantiomer is indeed benign.

As mentioned previously, males of *E. convergaria* were attracted in field surveys by a variety of homologs of the most attractive components. Experiments confirmed this lack of specificity, as specimens were readily captured in traps baited with 1 : 1 blends of the C_{18} or C_{20} trienes in combination with the respective 6*S*,7*R*-epoxides (Table 2). In fact, this insect was often caught in traps set for other species when the bait contained a triene and a *cis*-6,7-epoxydiene.

We conclude that a two-component blend of 3*Z*,6*Z*,9*Z*-19 : H with 3*Z*,9*Z*-6*S*,7*R*-epoxy-19 : H, in an approximate ratio of 1 : 1, is a sex attractant for male moths of this species. However, there is a remarkable amount of leeway in the

TABLE 2. CAPTURES OF *Eufidonia convergaria* IN TRAPS BAITED WITH MIXTURES OF C_{18} OR C_{20} TRIENES AND EPOXIDES[a]

Lure composition (μg)	Total males captured[b]
3*Z*,9*Z*-6*S*,7*R*-epoxy-18 : H (500)	0
3*Z*,9*Z*-6*S*,7*R*-epoxy-18 : H (475) + 3*Z*,6*Z*-9*Z*-18 : H (25)	1b
3*Z*,9*Z*-6*S*,7*R*-epoxy-18 : H (400) + 3*Z*,6*Z*-9*Z*-18 : H (100)	4b
3*Z*,9*Z*-6*S*,7*R*-epoxy-18 : H (250) + 3*Z*,6*Z*-9*Z*-18 : H (250)	74a
3*Z*,9*Z*-6*S*,7*R*-epoxy-20 : H (500)	0
3*Z*,9*Z*-6*S*,7*R*-epoxy-20 : H (475) + 3*Z*,6*Z*-9*Z*-20 : H (25)	0
3*Z*,9*Z*-6*S*,7*R*-epoxy-20 : H (400) + 3*Z*,6*Z*-9*Z*-20 : H (100)	4a
3*Z*,9*Z*-6*S*,7*R*-epoxy-20 : H (250) + 3*Z*,6*Z*-9*Z*-20 : H (250)	43a
3*Z*,9*Z*-6*S*,7*R*-epoxy-20 : H (100) + 3*Z*,6*Z*-9*Z*-20 : H (400)	23a
3*Z*,9*Z*-6*S*,7*R*-epoxy-20 : H (25) + 3*Z*,6*Z*-9*Z*-20 : H (475)	0
3*Z*,6*Z*-9*Z*-20 : H (500)	0

[a] Enantiomeric excesses of 3*Z*,9*Z*-6*S*,7*R*-epoxy-18 : H and 3*Z*,9*Z*-6*S*,7*R*-epoxy-20 : H were 88.2% in both cases.

[b] Replicated traps ($N = 3$) containing C_{18} compounds were set out June 10–29, 1985; replicated traps ($N = 2$) containing C_{20} compounds were set out June 29–July 18, 1985. Values followed by the same letter are not significantly different ($P > 0.05$).

specificity of the attractant chemicals with respect to chain lengths, as the insect also responded readily to the C_{18} and C_{20} homologs. Furthermore, the GC-EAD and GC-MS data from the pheromone gland extracts, in conjunction with the field trapping results, strongly suggest that 3*Z*,6*Z*,9*Z*-19:H and 3*Z*,9*Z*-6*S*,7*R*-epoxy-19:H are female-produced sex pheromone components for this species.

To date, no specific behavioral antagonists have been identified for this species. However, indirect evidence suggests the existence of at least one antagonist, as no *E. convergaria* males were caught prior to 1984, which was the first year that the individual epoxide regioisomers were set out instead of the combined monoepoxide mixture (CME-19:H). This suggests that one or both of the other regioisomers in the CME-19:H mixture are behaviorally antagonistic. Behavioral antagonism by regioisomers and stereoisomers of chiral unsaturated epoxide sex attractants and sex pheromones of this type also has been noted in several other moth species (Millar et al., 1989a,b).

Caripeta angustiorata male moths were first caught in 1984, when six of seven specimens taken were attracted to baits containing 3*Z*,9*Z*-*cis*-6,7-epoxy-19:H as a major component. In EAG studies, a male antenna gave large responses to CME-19:H (~4 mV), and when tested alone, the 3*Z*,9*Z*-*cis*-6,7-epoxy-19:H regioisomer gave much larger responses than did the 3,4- or 9,10-epoxide regioisomers. There were no significant responses to diene or triene hydrocarbons.

GC-EAD runs were conducted with standards and with an extract of pheromone glands from a single field-collected female of unknown mating status (Figure 2). With the standards, a single strong antennal response was obtained to 3*Z*,9*Z*-*cis*-6,7-epoxy-19:H, with much smaller responses to 3*Z*,9*Z*-*cis*-6,7-epoxy-20:H, 3*Z*,9*Z*-*cis*-6,7-epoxy-18:H, and 3*Z*,6*Z*-*cis*-9,10-epoxy-18:H.

In the GC-EAD traces of the female extract (Figure 2, bottom), a single small but definite response was seen from the male antenna, at a retention time corresponding exactly to 3*Z*,9*Z*-*cis*-6,7-epoxy-19:H. There was also a significant peak in the FID trace of the female extract that corresponded exactly in retention time with 3*Z*,6*Z*,9*Z*-19:H, but, as with the standards, there was no observable antennal response to this compound.

Further field tests (Table 3) suggested that 3*Z*,6*Z*,9*Z*-19:H is required to get attraction in the field, and that the attractive epoxide enantiomer is 3*Z*,9*Z*-6*R*,7*S*-epoxy-19:H.

Male moths of the noctuid species *Rivula propinqualis* were first captured in low numbers in 1983, primarily in traps baited with CME-19:H and 3*Z*,6*Z*,9*Z*-19:H. A few specimens also were taken in 1984. EAG studies with standard compounds indicated that the male antennae gave strong responses (>3 mV) to CME-19:H and to 3*Z*,9*Z*-*cis*-6,7-epoxy-19:H, with much smaller responses (<1 mV) to 6*Z*,9*Z*-*cis*-3,4-epoxy-19:H, 3*Z*,6*Z*-*cis*-9,10-epoxy-19:H, and 3*Z*,6*Z*,9*Z*-19:H.

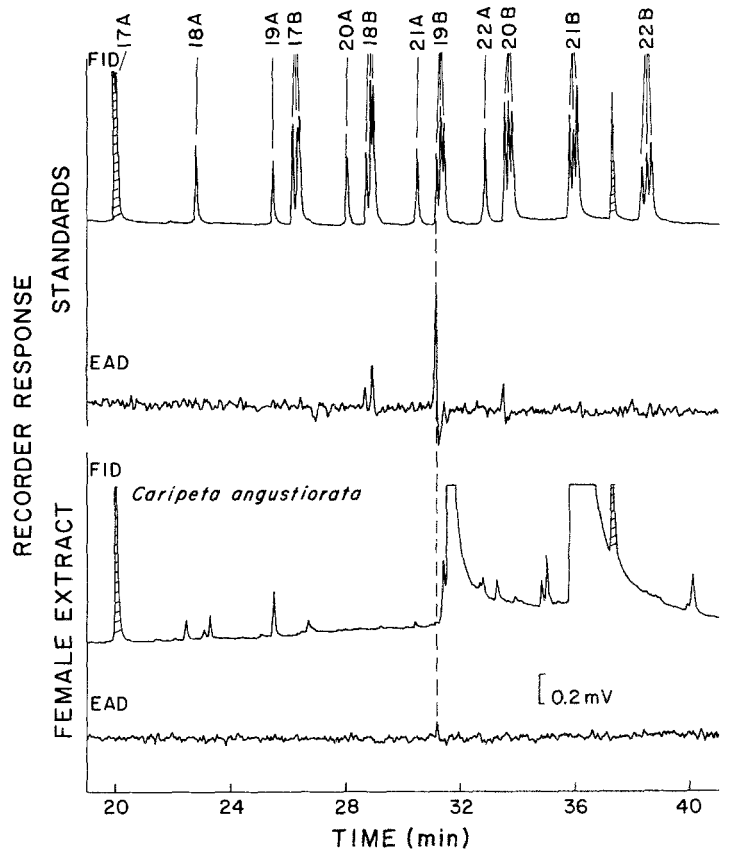


FIG. 2. Responses of *Caripeta angustiorata* male antennae in GC-EAD experiments. Upper pair of traces are in response to a mixture of C_{17-22} synthetic standards; lower pair are in response to an abdominal tip extract from a conspecific female. Number and letter designations of compounds are identical to those in Figure 1. Analyses were run on a 30-m DB-1701 column; epoxides eluted from the column in the order 6,7-, 3,4-, and 9,10-monoepoxydiene. Internal standards: 17:H and 24:H (shaded).

Survey traps in 1985 (replicated twice) indicated that a two-component blend of 3*Z*,9*Z*-6*S*,7*R*-epoxy-19:H with 3*Z*,6*Z*,9*Z*-19:H, in a ratio of approximately 1:10, was attractive to male moths (Table 4). The corresponding blend of the opposite enantiomer (3*Z*,9*Z*-6*R*,7*S*-epoxy-19:H) with 3*Z*,6*Z*,9*Z*-19:H caught no moths, demonstrating that the moths were specifically attracted by the blend of the triene with the 6*S*,7*R* epoxide enantiomer. The traps baited with 3*Z*,9*Z*-6*S*,7*R*-epoxy-19:H alone caught only one moth. Although lures containing 3*Z*,6*Z*,9*Z*-19:H as a single component were not included in the field

TABLE 3. CAPTURES OF *Caripeta angustiorata* IN TRAPS BAITED WITH BLENDS OF 3Z,6Z,9Z-19:H AND ENANTIOMERICALLY ENRICHED 3Z,9Z-*cis*-6,7-O-19:H[a]

Lure composition (μg)	Total males captured[b]
3Z,9Z-cis-6,7-epoxy-19:H (500) + 3Z,6Z,9Z-19:H (250)	0
3Z,9Z-*cis*-6,7-epoxy-19:H (250) + 3Z,6Z,9Z-19:H (250)	1
3Z,9Z-6*S*,7*R*-epoxy-19:H (225) + 3Z,9Z-6*R*,7*S*-epoxy-19:H (25) + 3Z,6Z,9Z-19:H (250)	0
3Z,9Z-6*S*,7*R*-epoxy-19:H (200) + 3Z,9Z-6*R*,7*S*-epoxy-19:H (50) + 3Z,6Z,9Z-19:H (250)	0
3Z,9Z-6*S*,7*R*-epoxy-19:H (50) + 3Z,9Z-6*R*,7*S*-epoxy-19:H (200) + 3Z,6Z,9Z-19:H (250)	10
3Z,9Z-6*S*,7*R*-epoxy-19:H (25) + 3Z,9Z-6*R*,7*S*-epoxy-19:H (225) + 3Z,6Z,9Z-19:H (250)	12

[a]Enantiomeric excess of 3Z,9Z-6*R*,7*S*-epoxy-19:H, 88.3%; 3Z,9Z-6*S*,7*R*-epoxy-19:H, 88.2% ee.
[b]Replicated traps ($N = 3$) were fielded June 29–July 17, 1985.

survey in 1985 due to the large number of other baits to be tested, in the previous two years, traps baited with 3Z,6Z,9Z-19:H alone caught no moths. Thus, the data suggested that 3Z,6Z,9Z-19:H and 3Z,9Z-6*S*,7*R*-epoxy-19:H were mutually synergistic.

An experiment using blends of 3Z,6Z,9Z-19:H with the epoxide enantiomers confirmed these data (Table 5). Blend ratios of 19:1 and 4:1 of 3Z,6Z,9Z-19:H with 3Z,9Z-6*S*,7*R*-epoxy-19:H were attractive to *R. propinqualis* males. Trap captures decreased if the proportion of epoxide to triene was increased further. The corresponding series of traps baited with 3Z,6Z,9Z-19:H and 3Z,9Z-6*R*,7*S*-epoxy-19:H did not capture a single moth, confirming the enan-

TABLE 4. CAPTURES OF *Rivula propinqualis* MOTHS IN SURVEY TRAPS BAITED WITH BLENDS OF 3Z,6Z,9Z-19:H AND ENANTIOMERICALLY ENRICHED 3Z,9Z-*cis*-6,7-EPOXY-19:H[a]

Lure composition (μg)	Males captured[b]
3Z,9Z-6*R*,7*S*-epoxy-19:H (500)	0
3Z,9Z-6*S*,7*R*-epoxy-19:H (500)	1b
3Z,6Z,9Z-19:H (500) + 3Z,9Z-6*R*,7*S*-epoxy-19:H (50)	0
3Z,6Z,9Z-19:H (50) + 3Z,9Z-6*R*,7*S*-epoxy-19:H (500)	1b
3Z,6Z,9Z-19:H (500) + 3Z,9Z-6*S*,7*R*-epoxy-19:H (50)	28a
3Z,6Z,9Z-19:H (50) + 3Z,9Z-6*S*,7*R*-epoxy-19:H (500)	1b

[a]Enantiomeric excess of 3Z,9Z-6*R*,7*S*-epoxy-19:H, 88.3%; 3Z,9Z-6*S*,7*R*-epoxy-19:H, 88.2% ee.
[b]Replicated traps ($N = 2$) were set out from April to October 1985 and were rebaited twice during that period. Moths were caught from July 24 to August 21. Values followed by the same letter are not significantly different ($P > 0.05$).

TABLE 5. CAPTURES OF *Rivula propinqualis* MOTHS IN TRAPS BAITED WITH BLENDS OF 3*Z*,6*Z*,9*Z*-19 : H AND ENANTIOMERICALLY ENRICHED 3*Z*,9*Z*-6*S*,7*R*-EPOXY-19 : H[a]

Lure composition (μg)	Total males captured[b]
3*Z*,6*Z*,9*Z*-19 : H (500)	0
3*Z*,6*Z*,9*Z*-19 : H (475) + 3*Z*,9*Z*-6*S*,7*R*-epoxy-19 : H (25)	14ab
3*Z*,6*Z*,9*Z*-19 : H (400) + 3*Z*,9*Z*-6*S*,7*R*-epoxy-19 : H (100)	20a
3*Z*,6*Z*,9*Z*-19 : H (250) + 3*Z*,9*Z*-6*S*,7*R*-epoxy-19 : H (250)	3b
3*Z*,6*Z*,9*Z*-19 : H (100) + 3*Z*,9*Z*-6*S*,7*R*-epoxy-19 : H (400)	2b
3*Z*,6*Z*,9*Z*-19 : H (25) + 3*Z*,9*Z*-6*S*,7*R*-epoxy-19 : H (475)	0
3*Z*,9*Z*,6*S*,7*R*-epoxy-19 : H (500)	0

[a] Enantiomeric excess of 3*Z*,9*Z*-6*S*,7*R*-epoxy-19 : H, 88.2%.
[b] Replicated traps ($N = 3$) were set out from July 18 to August 12, 1985. Values followed by the same letter are not significantly different ($P > 0.05$).

tiomeric specificity of the attraction. In addition, the traps baited with either 3*Z*,6*Z*,9*Z*-19 : H or 3*Z*,9*Z*-6*S*,7*R*-epoxy-19 : H alone caught no moths, confirming the mutual synergism of the two components.

DISCUSSION

Our efforts for the past several years have focused on the synthesis and field testing of a large number of triene hydrocarbons, monoepoxydienes, and related compounds as potential sex attractants and pheromone components for lepidopteran species. Our goal has been to explore the boundaries of structural diversity to be found in pheromones of these types and to determine how widespread these compounds are among lepidopteran genera and families.

As such, we have obtained data on sex attractants and pheromone components for approximately 100 geometrid, noctuid, and arctiid moth species. Because of this large number of species, it has not been possible to lab-rear individual species. Therefore, for the species described here, we have had to rely on field captures (e.g., by sweep netting or black-light trapping) to obtain female insects for analysis of pheromone glands. Despite numerous efforts, the number of female moths caught has been low, in part because of their cryptic and/or ephemeral nature. In addition, female moths obtained in this way were of unknown age and mating status, resulting in highly variable titers of components in their pheromone glands. For these reasons, we often have not been able to demonstrate whether attractant chemicals are indeed present in female pheromone glands, as was the case with two of the species reported here.

As seen in the previously published or submitted sections of this work

(Wong et al., 1985; Millar et al., 1987, 1989a,b), and as is well known for lepidopteran species in general, the best sex attractants often consist of a blend of components (Cardé and Baker, 1984), with synergistic rather than additive properties. The use of two or more components in an attractive blend is often crucial in maintaining a discrete, species-specific chemical communication channel (Cardé and Baker, 1984). Furthermore, as a number of sympatric species may utilize common components in their pheromone blends, many male moths are sensitive to and are behaviorally antagonized by (i.e., the attractive response is suppressed) the other components in the pheromone blend produced by a nonconspecific female. Most lepidopteran sex attractants or sex pheromones reported to date have been achiral. However, the sex attractant blends reported or discussed here illustrate that chirality can be an important factor in lepidopteran sex pheromone or sex attractant formulation.

All three species described here are attracted by blends containing the common component 3*Z*,6*Z*,9*Z*-19:H, which is not attractive alone to any species. However, 3*Z*,6*Z*,9*Z*-19:H in combination with a second component in a fixed ratio results in a species specific attractant. In all three cases, the second component is also not attractive alone. In other words, for all three species, both the common component and a more species-specific component are required in a specific ratio in order to obtain a significant behavioral response. The uniqueness of the chemical communication channel for each species may be further reinforced by behavioral antagonists.

For example, *E. convergaria* males were optimally attracted to a 1:1 blend of 3*Z*,6*Z*,9*Z*-19:H and 3*Z*,9*Z*-6*S*,7*R*-epoxy-19:H. The optimal blend for *R. propinqualis* also is composed of these two components, but in a ratio of approximately 10:1. In this case, the blend ratios limit cross-attraction (cf. Table 1 vs Tables 4 and 5).

C. angustiorata males were not attracted to sex attractant mixtures of the preceding two species, as the *C. angustiorata* attractant blend is a mixture of 3*Z*,6*Z*,9*Z*-19:H with the other epoxide enantiomer, 3*Z*,9*Z*-6*R*,7*S*-epoxy-19:H.

Indirect evidence suggests that 6*Z*,9*Z*-*cis*-3,4-epoxy-19:H and/or 3*Z*,6*Z*-*cis*-9,10-epoxy-19:H are behavioral antagonists of *E. convergaria* males, as no moths of this species were caught with blends of 3*Z*,6*Z*,9*Z*-19:H with the combined monoepoxides mixture prepared from 3*Z*,6*Z*,9*Z*-19:H. However, when the combined monoepoxides mixture was separated into its constituent regioisomers, blends of the 3*Z*,9*Z*-*cis*-6,7-epoxy-19:H regioisomer with 3*Z*,6*Z*,9*Z*-19:H were highly attractive, indicating that the removal of the other two regioisomers from the blend had eliminated the behavioral antagonist(s) that had previously suppressed attraction.

Several sympatric species were also attracted to blends of C_{19} epoxydienes and 3*Z*,6*Z*,9*Z*-19:H. For instance, males of the sympatric geometrid species *Probole amicaria* Herrich-Schäffer and *Anavitrinella pampinaria* Guenée, both of which have approximately the same flight period as *E. convergaria* and *C.*

angustiorata, are attracted to blends of 3*Z*,6*Z*,9*Z*-19:H + 3*Z*,9*Z*-*cis*-6,7-epoxy-19:H + 6*Z*,9*Z*-3*S*,4*R*-epoxy-19:H (8:1:10) and 6*Z*,9*Z*-*cis*-3,4-epoxy-19:H + 3*Z*,9*Z*-6*S*,7*R*-epoxy-19:H (19:1), respectively (Millar et al., 1990a,b). Males of a third geometrid species, *Sicya macularia* Harris, whose flight period overlaps slightly with that of *E. convergaria*, are attracted by a blend of 3*Z*,6*Z*,9*Z*-19:H + 6*Z*,9*Z*-*cis*-3,4-epoxy-19:H (1:9). The response of *S. macularia* males to otherwise attractive baits is completely blocked by trace amounts of either enantiomer of 3*Z*,9*Z*-*cis*-6,7-epoxy-19:H (Millar et al., 1990a,b). Thus, although all six of these species share common sex attractant components, cross-attraction is limited by a combination of differing blend ratios, missing components, and behavioral antagonists.

In light of the behavioral antagonism of *E. convergaria* by 6*Z*,9*Z*-*cis*-3,4-epoxy-19:H and/or 3*Z*,6*Z*-*cis*-9,10-epoxy-19:H, it is noteworthy that *E. convergaria* males respond readily to either the higher or lower homologs of the attractive compounds in the optimal sex attractant. Despite the competition for a narrow range of chemical communication channels using C_{19} compounds, as discussed above, this suggests that there may be relatively few sympatric species using C_{18} or C_{20} communication channels during the flight period of *E. convergaria*. In other words, *E. convergaria* males have become highly tuned to variations in the blend of C_{19} compounds, but there has apparently been little or no selection pressure to accurately distinguish changes in chain lengths.

It must be emphasized that the synthetic chiral epoxides used in this study were highly enantiomerically enriched (>88% enantiomeric excess), but not enantiomerically pure. The effects of the small amounts of enantiomeric impurities are unknown, but two possibilities must be considered. First, the attractive responses seen may have been due in part to the approximately 94:6 ratios of enantiomers in the enantiomerically enriched epoxides (i.e., enantiomeric synergism). Conversely, the small amount of enantiomeric impurity in an enantiomerically enriched compound could act as a behavioral antagonist. This may have contributed in part to the relatively poor trap captures seen with, for instance, *C. angustiorata*.

The chemotaxonomic aspects of the attractants are intriguing. On one hand, both geometrid species discussed here belong to the subfamily Ennominae, where several similar types of compounds have been reported as pheromonal or sex attractant components (Wong et al., 1985; Millar et al., 1990a,b). On the other hand, the trienes and 3*Z*,9*Z*-*cis*-6,7-epoxydienes described here extend across two insect families, the Noctuidae and the Geometridae. Overall, this suggests that speculations as to pheromone components for related species should be made with caution, since considerable diversity exists in the placement of functional groups and chain lengths of pheromone components, even within the same genus. A more complete description of the extent and limitations of structural variations for these types of pheromones has been discussed elsewhere (Millar et al., 1990a,b).

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CHEMICAL ANALYSIS OF TEMPORAL GLAND SECRETIONS COLLECTED FROM AN ASIAN BULL ELEPHANT DURING A FOUR-MONTH MUSTH EPISODE

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Abstract—The temporal glands, modified facial apocrine sweat glands unique to elephants, release collectable secretions during an unusual physiological state termed "musth" in the Asian bull elephant (*Elephas maximus*). Recently we began the characterization of the chemical components of musth, especially in the temporal gland secretions (TGS), and the examination of the role of such secretions as agents for chemical communication between elephants. The present study focuses on possible correlations between testosterone levels in the serum and temporal gland secretions. We were especially interested in possible qualitative and/or quantitative changes in volatile compounds as the testosterone levels varied during a discrete musth period. Quantitative changes in TGS and serum testosterone were determined by radioimmunoassay. Qualitative and semiquantitative changes occurring in volatile composition were studied by high-resolution gas chromatography (fused silica capillary column, on column injection). Compound identification was by nuclear magnetic resonance, gas chromatography–mass spectrometry, and gas chromatography internal standards. Twenty-three major compounds and a number of minor components were identified. Androgen concentrations were correlated with TGS-specific volatiles including benzoic acid, 2-nonanone, 5-nonanol, tetradecanoic acid, and decanoic acid. The latter two compounds and (*E*)-farnesol, a major component of African TGS, demonstrated an inverse relationship to T levels.

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Key Words—Temporal gland secretions, testosterone, dihydrotestosterone, *Elephas maximus*, volatiles, elephant, benzoic acid, 2-nonanone, 5-nonanone, 5-nonanol, tetradecanoic acid, decanoic acid, (*E*)-farnesol.

INTRODUCTION

The function of the temporal gland in Asian bull elephants (*Elephas maximus*) has intrigued scientists for decades. This gland, unique to elephants, is a modified apocrine sweat gland, located in the mid-cheek region (Fernando et al., 1963; Estes and Buss, 1976). Secretions from the gland have been implicated in several aspects of chemical communication (chemocommunication) in African as well as Asian elephants (Buss et al., 1976; Rasmussen et al., 1984; Rasmussen, 1988). The unusual physiological state of musth in the Asian bull elephant is characterized in part by the emission of secretions from the temporal glands (Jainudeen et al., 1972a; Schmidt, 1978); such secretions do not occur in the nonmusth condition. Chemical analyses of the temporal gland secretions (TGS) have demonstrated high testosterone levels (Rasmussen et al., 1984) and a variety of volatile chemical species (Rasmussen, 1988). Identification of species-specific chemicals in the TGS during musth and the changes, both qualititive and quantitative, that occur in the composition of volatile components during the progression of a musth period may help elucidate possible chemocommunicative roles for this gland.

Specific chemicals in the temporal gland secretions of Asian bulls in musth may affect behaviors such as male–male or female–male relationships, perhaps through pheromonal mediation. Previous studies suggested that mainly females respond to musth temporal gland secretions; predominantly flehmen responses were recorded to samples of TGS that had been frozen and then thawed prior to bioassay (Rasmussen, 1988). [In contrast, recent studies have demonstrated that avoidance responses by females and young calves occurred frequently to samples of fresh TGS (Rasmussen, Haight, and Perrin, unpublished).] As preliminary studies comparing the TGS volatiles from Asian bull elephants in musth and those from an Asian bull elephant whose serum testosterone had been artificially lowered during musth (Rasmussen, 1988) suggested that serum T levels might correlate with volatile compounds, we recorded, as described in this paper, the chemical changes occurring in the TGS during an entire musth episode in an individual captive Asian bull elephant maintained on a constant diet. Serum testosterone was assayed in randomly obtained samples. TGS samples were collected almost daily and testosterone measured.

Concurrently, volatile components from the temporal gland secretions were identified and described. We chose to analyze components of TGS that are less volatile than the headspace volatiles for several reasons. (Headspace compo-

nents refer to those compounds that, in a closed chamber, volatilize and subsequently can be directly analyzed by gas chromatography (GC) or trapped in hexane prior to GC analysis.) First, our observations had indicated that components of less volatility are important in elephant-to-elephant communication involving flehmen responses and palatal pit area contact responses (Rasmussen, 1988), and second, we lacked, at the time of this study, the equipment and personnel time for headspace analyses. That an investigation of the moderate volatiles might be instructive was suggested by the flehmen responses of the female elephants, especially their strong responses to samples containing cholesterol (Rasmussen 1988).

Selected samples were analyzed by gas chromatography–mass spectrometry (GC-MS). This selection was based on the serum and TGS testosterone levels and the variety of volatiles demonstrated by GC. Direct or inverse quantitative relationships between androgen levels and the concentrations of several specific TGS volatiles were noted over the time of the musth period.

METHODS AND MATERIALS

Procurement of TGS Samples

Biological samples were obtained from a 22-year-old Asian bull (Tunga) housed at the Washington Park Zoo (WPZ) facilities in Portland, Oregon. One hundred one sequential TGS samples were collected from this bull at 24-hr intervals during a single four-month-long musth episode in 1986 (June 3–October 7, 1986). The total time for the musth period was 127 days: 87 days of continual secretion, followed by a 10-day hiatus, and then 30 additional days of secretion. Although the procurement of daily TGS was dangerous, it was accomplished with relative safety because of the WPZ's restraining crush, which limited bull movement during sample collection (Rasmussen et al., 1984; Acknowledgments). Specially designed wide-bore Erlenmeyer flasks with flattened openings were placed directly over the cleaned orifice of the temporal gland and pressure was firmly applied to obtain the secretion. Sufficient pressure was applied to ensure that the sample secretions were ejected into the flask. This simulated the effect of behaviors normally exhibited by musth bulls in captivity and in the wild, during which they shove their heads against objects with sufficient force to eject material from the gland 0.5–1.0 m. Samples were all fresh, flowing secretions. The bull soon learned to associate no unpleasantness with the procedure, and samples were obtained routinely. Samples were frozen in 1-ml aliquots in liquid nitrogen and stored at $-20°C$ until analysis. Whenever possible, concurrent serum samples were obtained by the method described by Hess et al. (1983). All serum and TGS samples were obtained between 9–11 AM.

Sample Analyses

Radioimmunoassay of T and DHT. Serum and TGS aliquots (500 μl) were extracted with redistilled diethyl ether and the extract sequentially chromatographed on two different Sephadex LH-20 chromatographic columns prior to analyses by radioimmunoassay (RIA) (Resko et al., 1973, 1980). The first column (1.0 g LH-20, elution phase hexane–benzene–methanol, 62:20:13 v/v) separated neutral and phenolic steroids. The second column (2.5 g LH-20, elution phase, hexane–benzene–methanol, 85:15:5 v/v) isolated progesterone (P), dihydrotestosterone (DHT), and testosterone (T).

Extraction and chromatographic losses were monitored by adding known amounts of tritiated authentic steroids to the elephant serum or TGS and processing in parallel with the samples for assay. Respective recoveries following the final chromatographic step were DHT—72.0% and T—70.0%. Water blanks were also processed in parallel to provide solvent–method blanks for each steroid (DHT, 18.6 ± 9.8; and T, 3.5 ± 1.9 pg). Reported values were corrected for both procedural losses and method blanks before correcting for aliquots assayed. Each sample was diluted with 500 μl of ethanol after chromatography and assayed at three or four different volumes. The reported values are the average concentration calculated from aliquots whose values fell between the 5–95% binding limits of the standard curve following a logit-log transformation. Further details of the procedures are listed in Rasmussen et al. (1984).

Gas Chromatography. One-milliliter aliquots of TGS from each sample day were extracted by hexane, dichloromethane, acetonitrile, and chloroform. The dichloromethane fraction contained most of the organics, which were further studied; they were filtered through sodium sulfate for water removal prior to GC analysis.

Samples were analyzed by on-column capillary column GC. If, after initial analysis, few compounds were detected, the sample was concentrated 10-fold.

Prepared sample extracts (0.5 μl) were injected directly by silica needle onto a fused silica column (DB-1, 60 m), using helium as the carrier gas, at a flow rate of 30 cm/sec. Flame ionization detector (FID) was utilized with makeup gases of O_2 (40%) and N_2 (60%) mixture. The oven of the Hewlett-Packard 5790A gas chromatograph was programmed to initiate a warming sequence at 35°C, increasing at a rate of 6°/min. The sequence required 48 min for completion and attained a maximum temperature of 323°C.

Standards during the on-column capillary column GC analyses included compounds identified in TGS by GC-MS (Wheeler et al., 1982; Rasmussen et al., 1986; Rasmussen, 1988). Standards were utilized both separately and as internal standards. The areas of all peaks were determined using an HP 5792 integrator. All samples were run in triplicate; the data were analyzed statistically for run-to-run reproducibility of 3%.

GC-MS. After the screening analyses by GC, representative extracts from samples demonstrating different testosterone levels were further analyzed by GC-MS. While quantitative comparisons were obtained through peak integration routines (HP 5792), identification of the specific profile constituents was accomplished by GC-MS. Gas chromatographic conditions for the GC-MS analyses were identical to those used for the routine GC analyses. When necessary, trimethylsilyl derivatives were prepared. For the initial studies, we utilized a Finnegan model 4000 mass spectrometer interfaced with an InCOS data system. Subsequently, the analyses were performed on a VG 7070 E-HF double-focusing mass spectrometer system using electron impact ionization at 70 eV. The mass spectrometer was equipped with an 11/250 data system. The NBS library of chemical compounds was utilized in computer searches involving compound identification. Numerous peaks were resolved. However, in this study, only compounds resolved in the initial GC-MS analysis with a greater than 80% purity and meeting the standard of the computer matching criteria of 90% were further studied, and obvious contaminants were ignored. Criteria for authentication included GC retention times and mass spectrometry. All compounds in Tables 3 and 4 (below) were verified by matching spectra information and relative retention times with those of authentic compounds. Particular attention was paid to two compounds: (*E*)-farnesol, previously identified as a major constituent of African elephant TGS (Wheeler et al., 1982), and 4-methylphenol, identified in African elephant TGS, Asian bull elephant TGS, and urine extracts of both male and female Asian elephants (Rasmussen et al., 1986). Other phenols (4-ethylphenol, 2-*n*-propylphenol, and 4-*n*-nonylphenol) reported in the temporal gland secretion from captive Asian bull elephant TGS (Rasmussen, 1988) also were monitored, as were additional ketones, aliphatic acids, and alcohols.

RESULTS

Testosterone and Dihydrotestosterone

Serum. Although it was possible to obtain only a few serum samples during the 1986 musth episode, the nine serum samples collected had significantly higher testosterone (T) levels during the first part of the musth episode than toward the end. The T concentrations ranged from 12.71 ng/ml during late musth to 125.7 ng/ml during early musth (Table 1). During the first two months of the musth episode, the average serum concentrations were 70.4 ng/ml ($\pm$13.5), $N = 6$, declining in a single sample during the third month of musth to 26.7 ng/ml, with the minimal level of 12.7 ng/ml achieved during late musth (fourth month). DHT levels in the serum declined from 7.98 to 1.41 ng/ml over the same time period. Serum data expressed as T/DHT ratios from this discrete

TABLE 1. CHANGES IN LEVELS OF SERUM STEROID HORMONES OF AN ASIAN BULL ELEPHANT DURING MUSTH

Days in musth episode	Testosterone (ng/ml)	Dihydrotestosterone (ng/ml)	Ratio T/DHT
Day 16	125.70	7.98	16.0
Day 24	51.83	3.70	14.0
Day 31	39.8	2.95	13.6
Day 42	42.54	3.55	12.0
Day 53	89.35	5.58	16.0
Day 59	70.87	3.54	20.0
Day 72	73.80	3.11	23.7
Day 82	26.67	3.33	8.0
Day 110	12.71	1.41	9.0
Mean	59.25	3.91	14.7
SE	11.5	0.62	4.9
Range	12.71–125.7	1.41–7.98	8.0–23.7

musth episode exhibited differences related to the time into the musth period, with ratios varying from 23.7 to 8.0.

Temporal Gland Secretions. Testosterone levels in the temporal gland secretions varied from 13.6 ng/ml to a high of 2.7 μg/ml (Table 2). Dihydrotestosterone levels varied from 3.28 ng/ml to 2.9 μg/ml (Table 2).

Temporal Changes in TGS-T and DHT during Musth. Notably, TGS testosterone levels were significantly higher during the initial phase of the musth episode than in the latter stages (Table 2). Average T levels in the TGS for the first two months (June and July, days 1–59) were 921.6 ng/ml; TGS-T was low for several days between the two clusters of high TGS-T levels, June 15–26, days 13–24, and July 14–31, days 42–59. During the third month (August, days 60–87) TGS-T level averages were 162.0 ng/ml and during the last month of the musth episode (September and October, days 88–117), 36.6 ng/ml (Table 2). During the first half of the musth period, 87% of the samples exhibited T levels above 300 ng/ml. During the latter half of the musth episode, 92% were lower than 300 ng/ml.

DHT levels exhibited similarly high values during the first half of the musth period (Table 2). Mean values from June and July, days 1–59, and the first half of August, days 60–75 (1007.4 ng/ml), were significantly higher than values from the next month (177.1 ng/ml) and the last month (75.9 ng/ml) (Table 2). DHT values were often higher than T levels (Figure 1).

T/DHT Ratio in TGS. T and DHT were generally elevated or lowered to the same degree throughout the four-month musth episode, resulting in T/DHT ratios that varied only between 0.16 and 2.8, except for two peaks of high T/

TABLE 2. SUMMARY OF TEMPORAL GLAND SECRETION STEROID HORMONE LEVELS DURING 1986 MUSTH EPISODE, JUNE–OCTOBER

	Testosterone (ng/ml)	Dihydrotestosterone (ng/ml)
First two months		
June 3–July 31		
Day 1–Day 59		
59 days, 45 samples		
Mean	921.6	1007.4
SE	91.9	95.0
Range	282.9–2781.3	128.6–2949.2
Third month		
August 1–August 28		
Day 60–Day 87		
28 days, 28 samples		
Mean	162.0	177.1
SE	29.1	33.1
Range	24.2–504.9	24.6–619.5
Last month		
September 8–October 7		
Day 88–Day 117		
29 days, 28 samples		
Mean	36.6	75.9
SE	4.3	11.1
Range	13.6–104.8	12.1–262.7

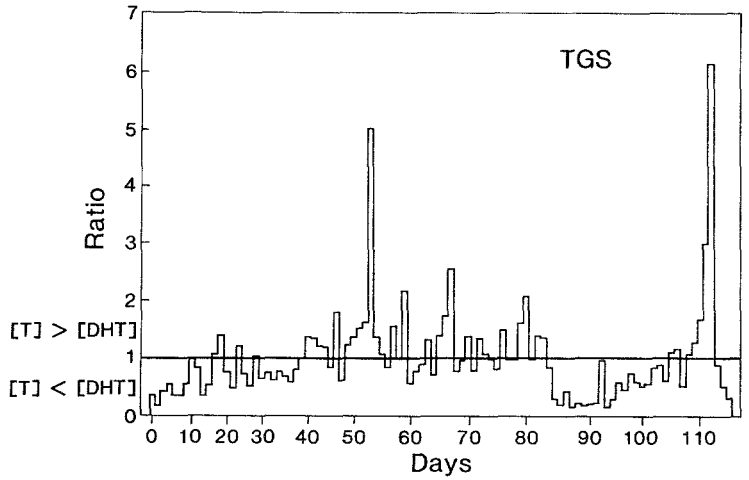


FIG. 1. Ratio of testosterone (T) to dihydrotestosterone (DHT) in the temporal gland secretions of an Asian bull elephant during a four-month musth episode. [T] > [DHT] means T concentration greater than DHT; [T] < [DHT] means DHT concentration greater than T. The solid line indicates the ratio of 1 or when the concentrations are equal. The horizontal axis is the days into the musth period.

DHT ratios (Figure 1). These TGS T/DHT ratios were lower compared to serum T/DHT ratios (8.0–23.7) (Tables 1 and 2).

Highest TGS/S ratios of testosterone were recorded between days 24 and 59 of the musth episode. This period of very high TGS-T levels also demonstrated the highest concentrations of 5-nonanol and 2-nonanone, as well as several other ketones. Several phenols were also relatively high just prior to this period (Figure 2).

Gas Chromatography and Mass Spectrometry

Dichloromethane extracted the most volatiles when compared to other solvents tested using capillary column gas chromatography, and only these results are reported.

GC-MS analysis was conducted on 30 extracts of samples of Asian bull elephant temporal gland secretion. Twenty-three major compounds and 16 minor compounds were identified. Table 3 lists all the compounds identified, together with their molecular weight, molecular ion, and mass ions in order of GC retention time. Eight compounds were detected in all musth TGS samples: 5-nonanol or 2-nonanol, 4-methylphenol, phenylacetic acid, 2-nonanone, octanoic acid, 4-*n*-nonylphenol, tetradecanoic acid, and testosterone (Table 4). Fifteen compounds were identified in more than five samples: phenol, benzoic acid, phenylpropionic acid, 2-hydroxyacetophenone, hexanoic acid, 3-octen-2-one, 4-ethylphenol, decanoic acid, 4-hexenoic acid, 3-nonen-2-one, pentadecanoic acid, hexadecanoic acid (palmitic acid), (*E*)-farnesol (3,7,11 trimethyl-1,6,10 dodecatrien-3-ol), dotriacontanol, and 3-ethyl-3-hydroxy-5-α-androstan-17-one.

The volatiles, as separated on GC during four representative periods during the musth episode, are depicted in Figure 2. These four selected composite chromatograms are representative of TGS samples containing various T concentrations during the progression of the musth period. Fluctuations of note in concentrations of TGS components are apparent. This especially includes various phenols, several alcohols (predominantly farnesol), several aliphatic and carboxylic acids, ketones, and steroids.

DISCUSSION

The musth period of Asian bull elephants is characterized by radical behavioral changes, including an increase in aggressiveness and dominance displays (Schmidt, 1978, 1989), and by physiological and biochemical events including temporal gland secretions, elevated serum testosterone (Jainudeen et al., 1972b; Rasmussen et al., 1984), and TGS testosterone levels in the microgram range (Rasmussen, 1988). Distinct differences in the TGS volatiles from Asian bulls in musth and a bull whose musth cycle was artificially terminated for manage-

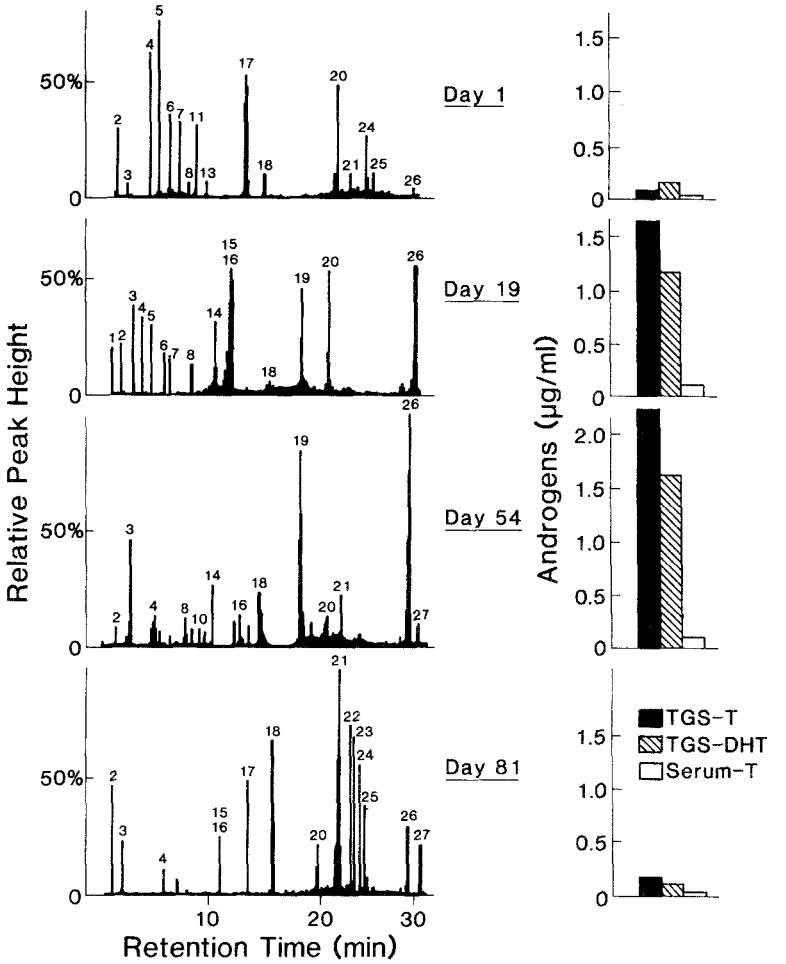


FIG. 2. Depicted in the major graph on the left are the fluctuating concentrations of volatiles in temporal gland secretions of an Asian bull elephant during a discrete musth period. (peak 26, day 54 (graph 3) is set at 100%). The four graphs are composite chromatograms combining specific separation runs. Compounds include: 1, cyclohexanone; 2, phenol; 3, 5-nonanol; 4, 4-methylphenol; 5, benzoic acid; 6, phenylacetic acid; 7, phenylpropanoic acid; 8, 2-hydroxyacetophenone; 10, 2-nonanone; 11, octanoic acid; 13, 3-octen-2-one; 14, 4-ethylphenol; 15, 2-*n*-propylphenol; 16, 4-*n*-propylphenol; 17, decanoic acid; 18, 4-hexenoic acid; 19, 3-nonen-2-one; 20, 4-*n*-nonylphenol; 21, tetradecanoic acid; 22, pentadecanoic acid; 23, hexadecanoic acid; 24, farnesol; 25, dotriacontanol; 26, testosterone; 27, 3-ethyl-3-hydroxy-5α-androstan-17-one. The smaller graph on the right depicts the concentrations of temporal gland secretion testosterone (TGS-T), temporal gland secretion dihydrotestosterone (TGS-DHT), and serum testosterone (Serum-T).

TABLE 3. COMPOUNDS DETECTED BY GC-MS IN MALE ASIAN ELEPHANT[a] TEMPORAL GLAND SECRETION

Compound	RT	MW	*m/z* (relative abundance)
Dimethyl disulfide	7	94	94(100), 79(74), 47(60)
Dimethyl sulfone	7.3	94	94(50), 79(100)
Cyclohexanone	7.5	98	98(22), 70(30), 69(30), 55(100), 45(58)
Trimethylcyclohexane (1,3,5)	9.0	126	126(20), 111(50), 69(100), 57(70), 55(75), 41(80)
PHENOL	10	94	94(100), 66(16), 65(14), 39(8)
5-NONANOL	11	144	144(4), 87(65), 69(100), 41(35)
2-Nonanol	11	144	144(4), 69(10), 55(10), 45(100)
4-METHYLPHENOL	12.0	108	108(81), 107(100), 90(15), 79(43)
BENZOIC ACID	13.4	122	122(75), 106(5), 105(100), 77(70), 51(45), 50(40)
PHENYLACETIC ACID	13.8	136	136(35), 92(10), 91(100)
PHENYLPROPANOIC ACID	13.9	150	150(40), 105(10), 104(45), 95(5), 91(100)
2-HYDROXYACETOPHENONE	14.1	136	136(5), 122(15), 106(5), 105(100), 77(55), 51(10)
Toluene	14.3	92	92(50), 91(100), 64(10), 39(5)
2-NONANONE	14.4	142	142(5), 71(20), 59(20), 58(100), 57(15), 43(85)
5-Nonanone	14.4	142	142(10), 85(90), 58(75), 57(100), 41(40)
3-Nonanone	14.4	142	142(5), 114(35), 72(50), 58(80), 43(100)
HEXANOIC ACID	14.5	116	116(5), 87(10), 73(40), 60(100)
OCTANOIC ACID	15	144	144(2), 116(5), 101(10), 73(75), 60(100), 55(30), 43(50), 41(40)
3-OCTEN-2-ONE	15.3	126	126(15), 11(60), 55(100), 43(80), 41(45)
Cyclododecanone	15.5	182	182(5), 139(5), 11(25), 71(50), 58(50), 55(90), 42(100)
4-*n*-ETHYLPHENOL	16	122	122(31), 108(5), 107(100), 77(5)
2,3-Dihydroindole	16.5	119	119(60), 118(100), 117(20), 91(20)
2-*n*-Propylphenol	17	136	136(40), 121(100), 122(5), 107(15), 91(5), 77(7)
4-*n*-Propylphenol	18	136	136(35), 122(5), 121(100), 107(10), 79(45), 78(5), 77(35)
DECANOIC ACID	19	172	172(5), 129(40), 73(90), 71(30), 60(100), 58(45), 56(40), 44(50), 42(50)
4-hydroxyacetophenone	20	136	136(40), 121(100), 94(40), 65(36)
4-HEXENOIC ACID	21	114	114(50), 72(20), 69(30), 68(40), 60(50), 56(100), 41(50)
3-NONEN-2-ONE	23	140	140(5), 125(55), 98(30), 71(30), 55(100), 43(100), 41(35)
8-Nonen-2-one		140	140(3), 82(25), 71(30), 58(60), 43(100)
5-Nonen-2-one		140	140(3), 82(30), 60(30), 55(30), 43(100)
4-*n*-NONYLPHENOL (and isomers)	26	220	220(5), 192(5), 164(10), 149(45), 134(100), 135(10), 122(50), 107(55)
tridecanoic acid	27	214	214(10), 171(15), 129(30), 115(15), 73(100), 60(100), 57(50), 55(50), 43(80), 41(70)
TETRADECENOIC ACID	29	228	228(20), 185(20), 129(30), 73(100), 71(30), 69(30), 60(90), 57(55), 55(60), 43(85), 41(65)
PENTADECANOIC ACID	30	242	242(30), 198(15), 185(5), 129(30), 74(100), 71(30), 69(80), 60(90), 57(60), 55(60), 43(95), 41(80)
HEXADECANOIC ACID	30.5	254	254(20), 236(10), 84(40), 69(70), 55(100), 43(60), 41(90)

TABLE 3. Continued

Compound	RT	MW	*m/z* (relative abundance)
(*E*)-FARNESOL (3,7,11-trimethyl-1, 6,10-dodecatrien-3-ol)	29-31 -32	222	222(5), 207(2), 204(15), 191(10), 189(5), 162(10), 93(40), 69(100), 41(80)
DOTRIACONTANOL	34	466	97(10), 85(40), 71(50), 69(45), 58(100), 43(100)
TESTOSTERONE (17-β-hydroxy-4-androsten-3-one)	36	288	288(35), 246(40), 228(5), 204(20), 147(30), 125(100), 109(30), 107(35), 105(40), 95(20), 93(35), 91(50), 55(20), 41(55)
3-ETHYL-3-HYDROXY-(5-α)-ANDROSTAN-17-ONE	36	318	318(5), 300(10), 272(5), 218(5), 190(10), 161(20), 147(40), 135(40), 121(40), 119(40), 107(50), 105(80), 93(95), 91(100), 82(75), 80(80), 78(40), 67(70), 55(80), 53(40), 41(40)

[a] CAPITALS: compound in all TGS samples; CAPITALS: compound in many TGS samples; lower case: compound in some TGS samples; RT = retention time; MW = molecular weight.

ment reasons have previously been reported (Rasmussen, 1988). These variations were sufficient to suggest that changes in TGS volatiles might be correlated to changes in physiology and behavior during musth. Our intimate access to healthy and behaviorally normal bull elephants has afforded us a unique oppor-

TABLE 4. COMPOUNDS IDENTIFIED IN TGS ASIAN BULL ELEPHANTS IN MUSTH -ORDERED PER INCREASING RETENTION TIME-

In all TGS samples	In more than five TGS samples
5-Nonanol	Phenol
4-Methylphenol	Benzoic acid
Phenylacetic acid	Phenylpropionic acid
2-Nonanone	2-Hydroxylacetophenone
Octanoic acid	Hexanoic acid
4-*n*-Nonylphenol	3-Octen-2-one
Tetradecanoic acid	4-Ethylphenol
Testosterone	Decanoic acid
	4-Hexenoic acid
	8-Nonen-2-one
	Pentadecanoic acid
	Hexadecanoic acid
	Farnesol
	Dotriacontanol
	Testosterone
	3-Ethyl-3-hydroxy-5α-androstan-17-one

tunity to analyze the biochemical variations which characterize a normal musth period in an Asian bull elephant.

Of special interest were androgen levels. The temporal patterns of T and DHT during an individual musth episode were distinctive in both the temporal gland secretions and serum. In the TGS, the significantly higher T and DHT shown during the first half of the musth episode were striking. The range of TGS-T concentrations was wide (13.6 ng/ml–2.8 μg/ml). In the serum, wide-ranging T concentrations (12.7–125.7 ng/ml) were also characteristic. These musth serum values were of considerably wider range than previously reported values (Jainudeen et al., 1972b; Rasmussen et al., 1984). In addition, previously, Jainudeen et al. (1972a,b) reported variations in serum T levels during premusth, full musth, and postmusth sampling of about a dozen different bulls. Our report is the first to monitor the chemical changes which occur during a complete musth episode in a single Asian bull elephant.

DHT concentrations in both the serum and the TGS of Asian bull elephants in musth were remarkably high. Other reported exceptions to the characteristically low serum DHT in mammals include rhesus monkeys (Resko et al., 1980) and ferrets (Rieger and Murphy, 1977).

Bull Asian elephants in musth also have elevated serum T/DHT ratios as compared to other male mammals including humans, bats, and dogs (Fiorelli et al., 1976; Bernard, 1986; Tremblay et al., 1972; Folman et al., 1972). Superficially, these high ratios appeared similar to the relatively high ratios observed in some mammals during the breeding season or during rut. For example, mean T/DHT serum ratios in breeding male ferrets ranged from 1 to 4 (Rieger and Murphy, 1977) and in breeding badgers from 11.4 to 17.5 (Maurel et al., 1981). Examination of our data from three studies (this study; Rasmussen et al., 1984; Rasmussen, 1988) demonstrated that three Asian bull elephants, during five successive musth periods, had serum T/DHT ratios ranging from 8.0 to 58.3. [Ratios in nonmusth serum were generally twofold lower; toward the end of the long 1986 musth period, low ratios of 8.0 and 9.0 occurred in serum (Table 1).] Two significant differences appeared when the elephant data were compared with those derived from studies of rutting or breeding animals: serum T levels were strikingly high in the musth Asian bull elephant, and T/DHT ratios were high. In breeding badgers, the high T/DHT ratio occurred when both T and DHT concentrations were low (Maurel et al., 1981). By contrast, high total DHT levels in the elephant were coupled with the extremely high T levels to produce a high/high situation.

In the TGS a higher ratio of T/DHT was seen during the heaviest behavioral musth and time of maximum secretion, June 11–August 24, than during either very early musth or most days toward the end of the musth period (Table 2). TGS samples obtained before 1986 were biased as most were gathered during times of moderate to heavy musth.

In addition, the TGS/serum ratio of testosterone changed during the musth period, becoming very high during "heaviest" musth when the most unpredicable and aggressive behaviors occur, usually concurrently with copious excretion of odoriferous glandular products. The greatest variety and concentration of volatiles, as well as the highest levels of testosterone and dihydrotestosterone, were noted in temporal gland secretion at this time.

Concurrent analysis of the steroid hormones of serum and TGS, and of the organic-extractable volatile TGS components over time demonstrated some definitive changes in these chemical species as the T content fluctuated. Our TGS data suggest that steroid hormonal levels correlate with the composition and amounts of TGS volatiles, chiefly phenols, aliphatic acids, carboxylic acids, alcohols, and ketones during musth in Asian bull elephants. Three striking pattern changes in TGS volatiles were noted during the course of the musth episode: (1) a high proportion of benzoic acid and certain substituted carboxylic acids in early musth; (2) a predominance of ketones during the time of heavy musth (40–70 days); and (3) Similarity of patterns from very early musth and very late musth TGS, especially with regard to long-chain aliphatic acids and two higher alcohols: dotriacontanol and farnesol (Figure 2).

Such moderately volatile compounds deposited in the environment could serve as chemical communicators to neighboring elephants, perhaps affecting long-term relationships such as dominance hierarchy by conveying less immediate, longer-lasting information relative to the hormonal and/or physiological status of the depositor. Processing may occur, as with other stable organics, via the vomeronasal organ. Because of the frequency of flehmen responses by females to moderately aged (2–24 hr) or frozen/thawed TGS, we had hoped to identify organic-solvent-extractable molecules, perhaps steroidal in nature. No single compound appeared to dominate among the volatiles, and bioassays of identified compounds only demonstrated the novel substance response among both the bulls and the cows. Probably the bioactive compounds are either in very low concentration or exist in combinations or bioactivity resides in more volatile compounds.

Recent behavioral response data from both female and male Asian elephants revealed that olfactory responses and avoidance behaviors (defined as deliberate nonapproach to areas that in normal situations would be approached, such as piles of bananas or apples) were of higher frequency toward newly collected, fresh (less than 1 hr) TGS as compared to frozen/thawed TGS or to aged TGS (Rasmussen, Haight, and Perrin, unpublished); in contrast frozen TGS or 1-day-aged TGS elicited a higher proportion of flehmen responses among the total responses observed (Rasmussen, 1988). The more volatile, ephemeral compounds, probably received by the olfactory system, may serve as immediate informants of the presence of a nearby, potentially aggressive bull, as evidenced by the consistency and rapidity with which cows and calves avoid such odors.

Such observations support the concept of the temporal gland as a multifunctional gland.

At present it cannot be stated that T levels influence levels and composition of volatiles in Asian bull elephants in musth, but only that the concentrations of selected volatiles in the temporal gland secretions change concurrently with altered serum testosterone levels. More direct relationships between T levels, released volatiles, and aggression and dominance ranking have been described in pigs, where pheromones and androgens affected aggression (Parrott et al., 1985), and in male golden hamsters, where endogeneous androgens were associated with changes in dominance status (Drickamer et al., 1978). In mice, critical T-dependent, aggression-eliciting characteristics of urinary pheromones were identified (Duvall et al., 1978). In male pine voles a urinary volatile appeared to have an endocrine dependency (Boyer et al., 1989). Our data suggest, in a similar fashion, that the levels of certain volatiles may be under endocrine control and that some of the TGS volatiles of Asian bull elephants in musth may function in chemocommunication, perhaps in the context of conveying information to the older, dominant bulls that younger, mature bulls are present and perhaps ready to challenge, as suggested by Schmidt (1989). The variation seen in T and DHT levels and ratios over time during musth suggest these steroid hormones may either play a role in influencing the events of musth or reflect the chemistry of musth.

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ALLELOPATHIC AND AUTOTOXIC EFFECTS OF *Anastatica hierochuntica* L.

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Abstract—Laboratory experiments were undertaken to investigate the autotoxic effects of *Anastatica hierochuntica* and possible effects on five other desert plants: *Rumex cyprius*, *Trigonella stellata*, *Diplotaxis harra*, *Cleome droserifolia*, and *Farsetia aegyptia*. Seed germination, seedling growth, and cell division of all species tested were inhibited by the shoot aqueous extract of *A. hierochuntica*. A gradual increase in the percentage of prophase and decrease in the other mitotic stages as well as the mitotic index were observed with increasing extract concentration. At an extract concentration of 8% the mitotic index was reduced from the control by 55% in *C. droserifolia*, 54% in *T. stellata*, 45% in *F. aegyptia*, 43% in *A. hierochuntica*, and 35% in *R. cyprius*. The inhibitory substances are apparently released onto soil by repeated washing of the standing plants by rain and dew interception.

Key Words—Allelopathy, *Anastatica hierochuntica*, germination, seedling growth, mitotic index, desert plants.

INTRODUCTION

Higher plants have developed various means of defense against their associates; whenever this defense is chemical in nature, it is referred to as allelopathy (Molisch, 1937; Rice, 1984). The ecological significance of allelopathy is recognized in agricultural practices (Einhelling and Leather, 1988), vegetational pattern (Muller, 1966; Rice, 1977), succession (Wilson and Rice, 1968), plant productivity (Rice, 1984), and microbial populations in soil (Kaminsky, 1981).

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Hitherto, the study of allelopathy due to waterborne inhibitors has been extended to ecosystems where environmental conditions allow for regular leaching of plant inhibitors, for example, in the tropical and deciduous forests where there is high rainfall. In desert ecosystems, it might be expected that considerable competition for water may result in the development of allelopathy. However, allelopathy studies in natural desert plant communities of Egypt are still lacking.

In most cases, pure populations of *Anastatic hierochuntica* L. (Cruciferae) grow in runnels and exposed depressions. Although the soil is fully exposed to the sun and receives ample runoff water, no other plants grow within *A. hierochuntica* patches (Friedman and Stein, 1980; Hegazy, 1990). It seems that water-soluble inhibitors are responsible for this particular ecological situation. These inhibitors are released onto soil by repeated washing of the standing dry plants by rain and dew interception or by microbial decomposition of plant litter and root exudates.

The present study was conducted to determine: (1) whether aqueous extracts of *A. hierochuntica* are autoinhibitory to seed germination, seedling growth, and cell division, as well as the possible allelopathic effects on five other associated species; and (2) whether such an inhibitory effect, if present, is operative in nature.

METHODS AND MATERIALS

Preparation of Aqueous Extract. Dry skeletons of *A. hierochuntica* were collected from Wadi Hagoul, eastern desert, Egypt. A stock aqueous extract (10% w/v) was obtained by soaking the ground air-dried skeletons in distilled water at 30°C for 24 hr with occasional shaking. During the soaking period, there were no signs of the development of any microflora or change in color and odor of the extract. The infusion was decanted and filtered through a Buchner funnel lined with double layer of Whatman No. 1 filter paper. The extract was then purified by centrifugation at 15,000 rpm for 15 min. The purified extract was adjusted to pH 6.8 with 1 N HCl (Rice, 1972). Aliquots for germination and seedling growth tests were made by diluting known volumes of the stock extract to concentrations of 8, 6, 4, and 2% (v/v extract–distilled water). The osmotic pressure of the aliquots was low (range 0.02–0.05 MPa) and hence discounted as a factor affecting germination and growth of the test species (Bell, 1974).

Effects of Aqueous Extract on Germination, Seedling Growth, and Cell Division. Germination was carried out in covered glass Petri dishes (9 × 1.5 cm) on two layers of Whatman No. 1 filter paper. In every dish 50 seeds and 3 ml of the extract were used. Distilled water was used for the control treatment.

The dishes were incubated at 25°C day and 15°C night temperatures. Five replicates per treatment per species were used.

The germination value (*CV*), i.e., the expression of speed and totality of germination, and their interaction as a single numerical value (Czabator, 1962) was estimated as follows: $GV = PV \times MDG$, where *PV* is the peak value, and *MDG* is the mean daily germination. Tests were terminated after two weeks when cumulative germination leveled off in all treatments. The average length of roots and shoots of the seedlings was measured at the end of the two week germination period.

For cytological investigations, the root tips were cut and fixed in 3:1 ethanol–acetic acid (v/v) for 24 hr. They were then stored in 70% ethanol. Squash preparations were made of root tips, hydrolyzed in 1 N HCl at 60°C for 6 min and then stained with Feulgen's reagent (cf. Mansour, 1984). The number of dividing and nondividing cells were counted in 50 microscopic fields. The mitotic index [MI = (number of dividing cells/total number of counted cells) × 100] and percentage of different mitotic stages were estimated.

Effect of Field Soil on Germination and Seedling Growth. This experiment was conducted to determine whether allelopathic compounds were released by *A. hierochuntica* onto soil and their likely effect on germination and seedling growth. Soil to a depth of 20 cm was collected in February (rainy season) and September (dry season) from the natural habitat supporting populations of *A. hierochuntica* (test stand). Similar samples were taken from an adjacent area (control stand) with sparse growth of *Cleome droserifolia*, *Farsetia aegyptia*, *Diplotaxis harra*, *Trigonella stellata*, and *Rumex cyprius*.

The soil samples were air-dried and passed through a 2-mm sieve to separate litter and gravel. The air-dried sieved soil was spread in a 1-cm-deep layer in covered glass Petri dishes and moistened with distilled water to its full water-holding capacity. Fifty seeds were sown at a depth of 0.5 cm, then the dishes were incubated at 25°C day and 15°C night temperatures. Five replicates per species were used. The seedlings appeared within one week, and the experiment was terminated after three weeks. The germination value and the average length of roots and shoots of the seedlings were determined at the end of the three-week period.

Soil from both test and control stands was analyzed for pH, total soluble salts, texture, and mineral ions following Allen et al. (1974). Statistical analysis by *t* test was applied to verify the significance of differences between the control and test treatments.

RESULTS

Effect of Aqueous Extract on Germination and Seedling Growth. The aqueous extract caused significant inhibition in the germination value of the

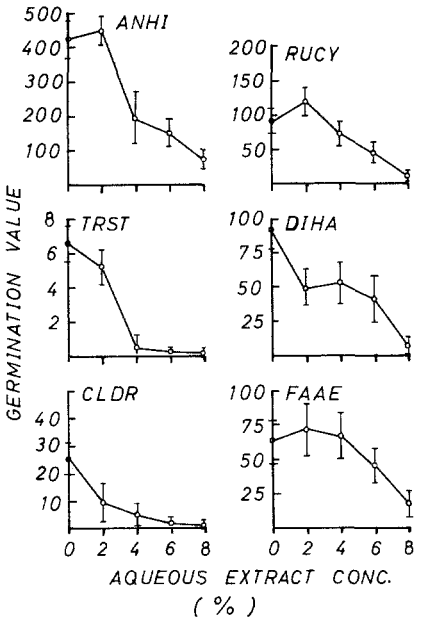


FIG. 1. Effect of aqueous extract of *Anastatica hierochuntica* (ANHI) on its germination value and possible effects on *Rumex cyprius* (RUCY), *Trigonella stellata* (TRST), *Diplotaxis harra* (DIHA), *Cleome droserifolia* (CLDR), and *Farsetia aegyptia* (FAAE). Vertical bars around the means are the standard deviations.

species tested (Figure 1). For *A. hierochuntica*, *R. cyprius*, and *F. aegyptia*, the inhibition was a function of concentration. There was an apparent promotion of germination at concentrations of 2%, followed by a decrease in the germination value at 4% and greater extract concentrations. The germination value of the remaining three species was inhibited regardless of the extract concentration. Germination of *T. stellata* was the lowest among all species with different concentrations of the extract.

Reduction of root and shoot growth was apparent in all species at 2% and greater concentrations of the aqueous extract (Figure 2). At the high concentration of 8%, there was no shoot development in *C. droserifolia* and *F. aegyptia*, while shoot growth exceeded root growth in *D. harra*. The root growth was more sensitive to the different extract concentrations than the shoot.

Effect of Aqueous Extract on Mitosis. The effects of *A. hierochuntica* extract on mitotic index (MI) and different mitotic stages of its own and other species are shown in Table 1. At the lowest concentration (2%), there were no significant differences in MI from the control treatment. This was true for all species tested. Generally, the MI decreased with the increase of aqueous extract

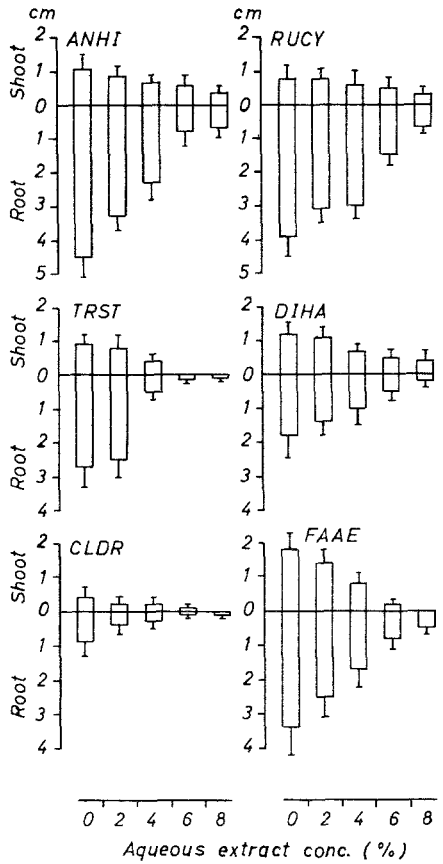


FIG. 2. Effect of aqueous extract of *Anastatica hierochuntica* (ANHI) on the seedling growth of RUCY, TRST, DIHA, CLDR, and FAAE. See Figure 1 for explanation of symbols.

concentration. At the highest concentration of extract used (8%), the MI was reduced from the control by 55% in *C. droserifolia*, 54% in *T. stellata*, 45% in *F. aegyptia*, 43% in *A. hierochuntica* and 35% in *R. cyprius*.

In all treatments for the six species tested, there was a tendency to increased percentage of cells in prophase with increased extract concentration. The opposite was true for cells in metaphase, anaphase, and telophase stages (Table 1). Regarding the relationship among different mitotic stages, the different concentrations of the extract did not significantly affect the sequence (percentage of each stage with respect to the other stages) of the four mitotic stages; its action was mainly in reducing the total number of dividing cells.

TABLE 1. EFFECT OF AQUEOUS EXTRACT OF *Anastatica hierochuntica* ON ITS OWN MITOTIC INDEX AND PERCENTAGE OF MITOTIC PHASES AND POSSIBLE EFFECTS ON FOUR OTHER SPECIES

Extract conc. (%)	Total counted cells	No. of dividing cells	Mitotic index ± SD	Mitotic stage							
				Prophase		Metaphase		Anaphase		Telophase	
				No. of cells	%	No. of cells	%	No. of cells	%	No. of cells	%
Anastatica hierochuntica											
0	1663	61	3.67 ± 0.46	22	36.07	18	29.51	10	16.39	11	18.03
2	1886	66	3.50 ± 0.42	26	39.40	18	27.27	10	16.15	12	17.18
4	1947	51	2.62 ± 0.36[a]	22	43.14	13	25.49	07	13.72	09	17.65
6	1724	50	2.90 ± 0.40	25	50.00	11	22.00	06	12.00	08	16.00
8	1665	35	2.10 ± 0.35[a]	19	54.28	07	20.00	04	11.43	05	14.29
Rumex cyprius											
0	1986	84	4.23 ± 0.45	32	38.10	28	33.33	11	13.09	13	15.48
2	1959	82	4.19 ± 0.45	34	41.46	25	30.49	11	13.42	12	14.63
4	1879	78	4.15 ± 0.46	38	48.72	22	28.20	08	10.26	10	12.82
6	2000	78	3.40 ± 0.43	41	52.56	21	26.92	07	08.97	09	11.55
8	2055	57	2.77 ± 0.36[a]	33	57.89	14	24.56	04	07.02	06	10.53

Trigonella stellata											
0	1505	65	4.32 ± 0.52	23	35.39	21	32.31	10	15.38	11	16.92
2	1692	61	3.61 ± 0.45	23	37.71	19	31.15	09	14.75	10	16.39
4	1608	47	2.92 ± 0.42[a]	20	42.55	14	29.79	06	12.77	07	14.89
6	1718	41	2.39 ± 0.37[a]	21	51.22	11	26.83	04	09.76	05	12.19
8	1603	32	2.00 ± 0.35[a]	18	56.24	08	25.00	03	09.38	03	09.38
Cleome droserifolia											
0	1652	99	5.99 ± 0.58	35	35.33	29	29.30	17	17.77	18	18.16
2	1690	77	4.56 ± 0.51	33	42.86	19	24.68	12	15.58	13	16.88
4	1531	59	3.85 ± 0.49[a]	30	50.85	14	23.73	07	11.86	08	13.56
6	1667	60	3.60 ± 0.46[a]	35	58.33	12	20.00	06	10.00	07	11.67
8	1700	46	2.71 ± 0.39[a]	28	60.87	09	19.56	04	08.70	05	10.87
Farsetia aegyptia											
0	1644	84	5.11 ± 0.54	33	39.29	27	32.14	11	13.09	13	15.48
2	1411	56	3.97 ± 0.52	25	44.64	16	28.57	07	12.50	08	14.29
4	1555	61	3.92 ± 0.49	31	50.82	15	24.59	07	11.48	08	13.11
6	1752	52	2.97 ± 0.41[a]	29	55.77	11	21.15	05	09.62	07	13.46
8	1559	44	2.82 ± 0.42[a]	28	63.64	08	18.18	03	06.82	05	11.36

[a]Significantly different from control at 0.05 level.

TABLE 2. SOIL FACTORS WITHIN AND OUTSIDE *Anastatica hierochuntica* STANDS[a]

	Within *Anastatica* stand	Outside *Anastatica* stand
pH	7.4 ± 0.3	7.1 ± 0.3
Total soluble salts (%)	00.320 ± 0.18[b]	00.392 ± 0.26
Mechanical analysis (%)		
Coarse sand	51.0 ± 4.6	54.2 ± 5.1
Fine sand	40.6 ± 2.8	38.4 ± 3.2
Silt	03.1 ± 0.6	02.5 ± 0.4
Clay	04.2 ± 0.5	03.5 ± 0.4
Mineral ions (%)		
Na	0.047 ± 0.009	0.058 ± 0.007
K	0.049 ± 0.006	0.041 ± 0.007
Mg	0.009 ± 0.002	0.017 ± 0.003
Ca	0.138 ± 0.018	0.115 ± 0.014
Fe	0.011 ± 0.003	0.008 ± 0.001
Cl	0.047 ± 0.008	0.062 ± 0.010

[a]Each value represents the mean plus minus standard deviation ($N = 5$).
[b]Significantly different at 0.05 level.

Effect of Field Soil on Germination and Seedling Growth. The physical and chemical soil properties were eliminated as factors affecting plant growth within and outside *A. hierochuntica* stands. Total soluble salts was the only soil factor tested that differed significantly between *A. hierochuntica* stands and the surrounding area (Table 2).

Soil collected from the control stand in February did not affect the germination and seedling growth of any of the test species significantly as compared with the soil collected in September (Table 3). Soil of the test stand collected in February and September had no effect on *A. hierochuntica*, *R. cyprius*, and *F. aegyptia* as compared with the control stand. Germination value and root growth of *T. stellata* and *C. droserifolia* were significantly inhibited by the soil of the test stand, while the shoots were not affected. Meanwhile, soil collected in September from the test stand significantly inhibited germination and growth of *D. harra*, while soil collected in February had no effect.

Within the test stand, time of soil collection did not influence germination and seedling growth of *A. hierochuntica* and *C. droserifolia* (Table 3). In *D. harra* and *F. aegyptia*, soils collected in September inhibited only root growth, while in *R. cyprius* and *T. stellata* both germination and root growth were significantly inhibited. The soils collected from within the test stand in February and September had no significant effect on shoot growth of all species tested.

TABLE 3. EFFECT OF FIELD SOIL FROM WITHIN AND OUTSIDE (CONTROL) AN *Anastatica hierochuntica* STAND ON ITS OWN SEED GERMINATION AND SEEDLING GROWTH AND ON FIVE OTHER SPECIES

Species	Date soil taken	Control stand			Test stand		
		GV	Root (cm)	Shoot (cm)	GV	Root (cm)	Shoot (cm)
Anastatica hierochuntica	February	23.2	3.8	0.9	22.5	3.7	0.9
	September	22.7	3.6	0.8	18.9	3.5	0.7
Rumex cyprius	February	19.1	3.1	0.7	19.6	2.9	0.8
	September	17.6	2.7	0.8	15.4[b]	2.4[b]	0.8
Trigonella stellata	February	03.9	2.2	0.4	00.8[a]	1.1[a]	0.3
	September	03.2	1.8	0.3	00.2[a,b]	0.7[a,b]	0.3
Diplotaxis harra	February	09.7	1.7	0.9	07.6	1.5	0.7
	September	08.2	1.7	0.9	05.3[a]	0.7[a,b]	0.5[a]
Cleome droserifolia	February	05.1	0.7	0.5	02.4[a]	0.4[a]	0.3
	September	04.2	0.7	0.4	01.8[a]	0.3[a]	0.3
Farsetia aegyptia	February	18.5	3.1	0.8	15.3	3.0	0.7
	September	18.4	3.0	0.6	14.1	2.3[b]	0.6

[a] Significantly different from control at 0.05 level.
[b] Significantly different from samples of February at 0.05 level.

DISCUSSION

Our results demonstrated the inhibitory properties of an aqueous extract of *Anastatica hierochuntica* on its seed germination, seedling growth, and cell division. The effect extended to five associated plant species. The basis for the inhibitory action of *A. hierochuntica* extract may be the role of specific chemicals. In phytochemical screening, the extract (Sayed, 1988) was found to contain glucoiberin, glucocheirolin, hydrocarbons, sterols, terpenes, flavonoids, and tannins. The water-soluble inhibitors appeared to be released and accumulated in the soil.

A major dispersal strategy of *A. hierochuntica*, known as "dispersal on the spot," results in regeneration in the vicinity of the parent plant (Friedman and Stein, 1980). This leads to a patchy dispersion of its individual plants. The presence of growth inhibitors in *A. hierochuntica* may have an ecological importance in being "autotoxic," i.e., inhibited by exudates or leachates from its own species, as well as inhibiting other associated species. This combined interference against itself and other species resulting from an allelopathic effect

could reduce both intra- and interspecific competition (McNaughton, 1968; Rice, 1972; Weaver and Klarich, 1977; Hegazy, 1990). This may be explained by the almost pure patches of *A. hierochuntica* that may remain in one place for several years.

The inhibitory effects of *A. hierochuntica* are species specific and vary with the extract concentration. Newman (1978) has suggested that if natural selection had favored ability in plants to reduce the growth of other species by allelopathy, plants should be, on average, more toxic to other species than to their own. Clearly, this applied to the species tested with *A. hierochuntica*. Such behavior often leads to a dominance of a particular species and failure of others under natural conditions (Alsaadawi and Rice, 1982). Therefore, the existence of *A. hierochuntica* in pure patches can be partially explained by its allelopathic effects on other species. Moreover, it has an autotoxic effect and thus lowers its populations, leading to increased successful establishment; that is, as seed germination and seedling growth are inhibited by allelopathic effects, competition will be reduced and chance for survival increases (Alsaadawi and Rice, 1982).

From a cytological point of view, little is known about the effect of aqueous plant extracts on the mitotic activity. Our results showed that the *A. hierochuntica* extract has a mitodepressive action, as demonstrated from the decreased mitotic index with the increase of extract concentration. This agrees with the findings of others (Moore, 1985; Bennett, 1987), who pointed out that cytological effects can exclude species from certain local flora, prevent expression of certain life-forms, and/or relate to particular timing of growth.

Several studies have illustrated that potentially phytotoxic substances may occur in the soil at concentrations high enough to cause inhibition of plant growth (Whitehead, 1964; Alsaadawi and Rice, 1982). Soil collected from the *A. hierochuntica* stand reduced seed germination and seedling growth compared with soil collected from the control stand. It is possible that the control soil had some allelopathic activity, and this could have masked some of the allelopathic activity of the soil under populations of *A. hierochuntica*. One may point out that the constant interception of moisture (rain and dew) onto the skeletons of *A. hierochuntica* and hence to the surrounding soil is sufficient to carry a regular supply of water-soluble inhibitors to the soil, where they remain in sufficient concentrations to inhibit growth of its own and of other associated species that spread their seeds near these plants. The inhibitors remain active at least until they are washed away by heavy rainfall.

In conclusion, *A. hierochuntica* may compete with other species by exerting allelopathic effects as well as by self-stimulation in the attainment of pure populations. Further work is needed on the identification and fate in soil of leachate constituents of dry and green plants of *A. hierochuntica.*

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MALE STERNAL PHEROMONE GLANDS IN ACANTHOSOMATID SHIELD BUGS FROM BRITAIN

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Abstract—Pheromone glands are present in the abdominal sternites of the pregenital region in the male adults in all British species of Acanthosomatidae. Secretory units are most numerous in the hawthorn bug *Acanthosoma haemorrhoidale* and maternal bug *Elasmucha grisea*, next most numerous in the birch bug *Elasmostethus interstinctus*, and least numerous in the juniper bug *Cyphostethus tristriatus*. The largest dorsal abdominal scent glands were found in *Cyphostetheus*. Ordinary dermal glands are also present. They are most numerous along the lateral margins of the abdomen. It is possible that Pendergrast's organ is the source of a female-produced pheromone. Secretory units were numerous in this organ in *C. tristriatus* female adults.

Key Words—Acanthosomatidae, *Acanthosoma haemorrhoidale*, *Cyphostethus tristriatus*, *Elasmostethus interstinctus*, *Elasmucha grisea*, Heteroptera, Pendergrast's organ, Pentatomoidea, pheromone glands, scent glands.

INTRODUCTION

The importance of semiochemicals in the population biology of plant feeding and predaceous bugs in the superfamily Pentatomoidea has become apparent only in recent years (Aldrich, 1988a,b). It seems to be the rule in the superfamily that the males emit pheromones that are attractive to the females (Zdarek and Kontev, 1975; Harris and Todd, 1980; Aldrich et al., 1987). The ability of parasitoids to utilize stink-bug pheromones as host location cues may explain the existence of a mating system in which the male provides the attractant signal and the female travels (Thornhill and Alcock, 1983; Aldrich, 1988a,b; Aldrich

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et al., 1989). Anatomical sources of pheromone include glandular elements in the abdominal sternites and the dorsal abdominal scent glands. The presence of sternal glands in adult male pentatomoids was first reported by Carayon (1981).

The family Acanthosomatidae in Britain contains the hawthorn bug *Acanthosoma haemorrhoidale* (L.), birch bug *Elasmostethus interstinctus* (L.), maternal bug *Elasmucha grisea* (L.), and juniper bug *Cyphostethus tristriatus* (Fabricius). The birch bug and maternal bug have been included in a list of invertebrates of economic importance in Britain (Seymour, 1979). In a previous paper, analyses by mass spectrometry of the secretions from the adult abdominal dorsal scent glands of all four British species of acanthosomatid have been reported (Evans, 1989; Evans et al., 1990). In this paper I describe the distribution of the male sternal pheromone glands and, for comparison, that of other ducted integumentary glands in the pregenital region of the adult abdomen.

METHODS AND MATERIALS

Fresh material for microscopic examination was obtained from local parks and woodlands. *Cyphostethus tristriatus* was collected from Lawson's cyprus (*Chamaecyparis*). Host trees were among those listed by Schaefer and Ahmad (1987) for the British acanthosomatids. Preserved museum specimens from Britain also were examined.

The abdominal sternites were prepared for microscopic examination by removal of soft tissues in hot caustic potash. Cuticular isolates were stained lightly with acid fuchsin and mounted in Balsam. Preparations of the abdominal tergites were similarly made to permit microscopic examination of the abdominal dorsal scent glands.

The cuticular ductules that drain the gland cells open between the epicuticular imprints of adjacent epidermal cells. External openings, which are not easily seen, are about 1 μm in diameter. However, there is a conspicuous dilatation at the basal free extremity of the ductule, and this is easy to distinguish in cuticular isolates. Diameters range between about 2 and 10 μm, and they stain intensely with acid fuchsin. The different types of gland could be distinguished by differences in cuticular morphology. Data on numbers and density of glands were obtained by counting basal dilatations.

RESULTS

There are at least four types of integumentary glands in the pregenital region of the abdomen in Acanthosomatidae.

1. Ordinary dermal glands are distributed generally throughout the integument. In *Cyphostethus tristriatus* they are densest in a 0.1-mm-wide band

extending along the lateral margins of the sternites and tergites (1400/mm^2) and least dense (150/mm^2) in the central areas of the sternites. They are present in the adults in both sexes. In the male adults, they are not excluded from territory occupied by the male sternal glands. The diameters of their basal dilatations are 2–3 μm, or appreciably less than those in the male pheromone glands (5–10 μm, according to the species). Thus they are less easily counted in cuticular isolates.

2. The male sternal glands (Figure 1) are restricted to the abdominal sternites of the pregenital region. They are most extensive in *Acanthosoma haemorrhoidale* (Figure 1A) and *Elasmucha grisea* (Figure 1F), less extensive in *Elasmostethus interstinctus* (Figure 1D), and least extensive in *Cyphostethus tristriatus* (Figure 1B). In *Acanthosoma haemorrhoidale* the density of sternal glands was about 3000/mm^2 and the total number 34,000. In *Cyphostethus tris-*

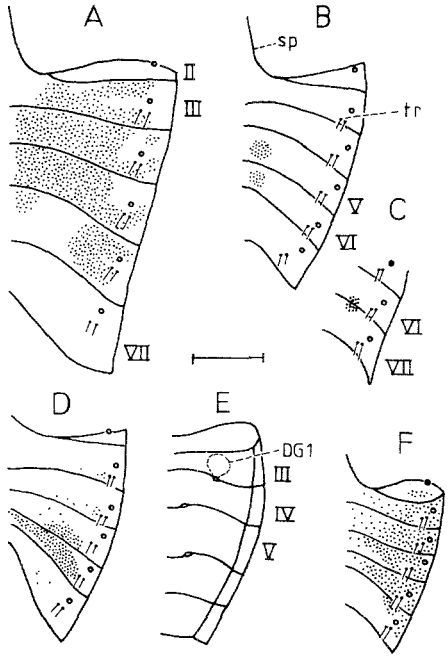


FIG. 1. (A) *Acanthosoma haemorrhoidale*, (B) *Cyphostethus tristriatus*, (D) *Elasmostethus interstinctus*, (F) *Elasmucha grisea*: ventral aspect of adult male abdomen showing distribution of sternal pheromone glands, stippled areas. (C) *Cyphostethus tristriatus*, location of Pendergrast's organ, stippled. (E) *Elasmostethus interstinctus*, dorsal view showing position of abdominal scent glands (DG1). Scale line = 1 mm. The abdomen bears paired trichobothria (tr) on sterna III to VII and a ventral spine (sp) on sternum III.

triatus, where they are mostly but not entirely confined to small patches in sternites V and VI, the total number was about 1660, or only one-twentieth of the total in *Acanthosoma haemorrhoidale*. The basal dilatations are about 10 μm in diameter in *Acanthosoma haemorrhoidale*, 7 μm in *Cyphostethus tristriatus*, and 5 μm in *Elasmostethus interstinctus*. In *E. interstinctus* the dilatations are unusually long (22 μm) and tapered.

There seemed to be no common underlying pattern to the distribution of the male sternal glands in the different species. Segmental abundances in *Acanthosoma haemorrhoidale* were greatest in sternites IV and V, greatest in *Cyphostethus tristriatus* in sternite V, and greatest in *Elasmostethus interstinctus* in sternite VI. *C. tristriatus* and *E. interstinctus* possess, perhaps, the remnants of a previously more extensive system of glands. Segmental abundances in *C. tristriatus* (one male, one side; segment number in parentheses) were 35 (II), 3 (III), 3 (IV), 300 (V), 253 (VI), 0 (VII). In *E. interstinctus* they were 0 (II), 25 (III), 13 (IV), 475 (V), 1079 (VI), 6 (VII).

3. Pendergrast's organ (location Figure 1C) is a setose organ to be found on the boundary of abdominal sternites VI and VII. It occurs in *Acanthosoma haemorrhoidale*, *Elasmostethus interstinctus*, and *Cyphostethus tristriatus* but not in *Elasmucha grisea* (Pendergrast, 1953; Southwood and Leston, 1959). It is present only in female adults. Carayon (1981) has examined this organ and noted the presence of ducted secretory units. I have examined cuticular isolates in *Cyphostethus tristriatus* and been able to follow the path of individual ductules through the cuticle of the body surface where they open separately by 1-μm-diameter openings between adjacent bristles. The total number of secretory units was about 1000. Pendergrast (1953) himself saw the cuticular ductules but did not appreciate their significance. He incorrectly linked them to the bases of the bristles. The maximum diameter of the cone-shaped dilatation of the ductule at its free basal end is in *Cyphostethus tristriatus* about 10 μm.

4. The abdominal dorsal scent glands lie below the surface. They are sac-like in form and open on the boundary between tergites III and IV (Figure 1E). They are present in the adults of both sexes. Differences between the sexes were not observed. The highest number of secretory units in the gland sac of one side was 494 in *Cyphostethus tristriatus*, the next highest 315 in *Elasmostethus interstinctus* and 292 in *Elasmucha grisea*, and the lowest 230 in *Acanthosoma haemorrhoidale*. Larger dorsal glands, it appears, are compensating for reduced male sternal glands in *C. tristriatus* and *E. interstinctus*. It has been reported that the secretions of the dorsal glands in the British acanthosomatids consist of a mixture of monoterpenes in proportions that differ in the different species (Table 1) (Evans, 1989; Evans et al., 1990).

In the adults, in all acanthosomatid species examined, vestiges of the undivided second and third dorsal glands, which function alone in the larvae, could be seen on the IV–V and V–VI segmental boundaries (Figure 1E).

TABLE 1. OCCURRENCE OF MONOTERPENES IN ABDOMINAL DORSAL SCENT GLANDS OF ACANTHOSOMATIDS FROM BRITAIN[a]

Component	Composition (%)			
	Ei	Eg	Ct	Ah
α-Pinene	<1	11		4
β-Pinene	17	18	28	74
Sabinene	47	33	25	
Myrcene	10	16	23	15
Limonene	11	14	17	7
Unknown	<1	<1		
γ-terpinene	13	7	7	

[a]Evans (1989) and Evans et al. (1990). Ei, *Elasmostethus interstinctus*; Eg, *Elasmucha grisea*; Ct, *Cyphostethus tristriatus*; Ah, *Acanthosoma haemorrhoidale*. Data from male adults.

DISCUSSION

Features of the acanthosomatid venter include the large mesosternal keel, the spine on abdominal sternum III (Figure 1B, sp), paired trichobothria on abdominal sterna III–VII (Figure 1B, tr), and frequently the deeply emarginated posterior border to sternum VII (Leston, 1953). An additional feature is supplied by the presence of sternal pheromone glands in male acanthosomatid adults (Figure 1).

Position and area development of the male sternal glands differed widely in the species studied. Factors conditioning differences can only be surmised at present. Large glands may enable males to attract females over greater distances (*Acanthosoma haemmorhoidale*). On the other hand, large glands may render males more conspicuous to predators such as parasitoids, which utilize pheromones as host location cues (Aldrich, 1988a,b; Aldrich et al., 1989).

Alternative mechanisms of bringing the sexes together may exist in some cases. Host tree volatiles rather than glandular secretions are perhaps aggregation promoting in *Cyphostethus tristriatus*. Differences among species may exist in physiological or behavioral response thresholds of females to male-produced pheromone.

It is possible that pheromone composition is regulated by segmental differences in sternal physiology. Segment-specific differences in character in the secretions from metamerically repeated glands have been described in *Oncopeltus fasciatus* (the dorsal scent glands: Games and Staddon, 1973) and *Manduca sexta* (Verson's glands: Horwath and Riddiford, 1988).

In *Cyphostethus tristriatus*, the near-midline position of the glands sug-

gests a mode of deposition of pheromone by rubbing the abdomen against the substratum.

The survey of the male sternal glands was extended to both sexes to include the dermal glands, Pendergrast's organ, and the abdominal dorsal scent glands. Pendergrast's organ is confined to acanthosomatid female adults. According to Southwood and Leston (1959), it is present in all the British species with the exception of *Elasmucha grisea*. The presence of numerous secretory units (*Cyphostethus tristriatus*) suggests that Pendergrast's organ is the source of a female-produced pheromone.

Dermal glands are widely present in the integument in both sexes. Their development is evidently independent of that of the male sternal glands. In *C. tristriatus*, densities were high in a strip extending along the lateral margins of the abdomen. Secretions from the dermal glands may play a role during courtship.

The abdomen in both sexes in all the British acanthosomatids contains well-developed dorsal scent glands (DG1: Figure 1E). The scent glands of *C. tristriatus* had the greatest number of secretory units. In this species the male sternal glands are relatively reduced.

The secretions of the scent glands have been analyzed and found to contain monoterpenes in proportions and combinations that differ in the different species (Evans, 1989; Evans et al., 1990, their Table 1). β-Pinene was dominant in *Acanthosoma haemorrhoidale*; sabinene was dominant in *Elasmostethus interstinctus*. The secretions probably combine several functions as olfactory repellents and attractants (Aldrich, 1988a,b). An alarm pheromone effect in larvae of *E. grisea* has been reported (Maschwitz and Gutmann, 1979).

Acanthosomatidae in Britain are of little economic importance as pests and have not been extensively studied. *Elasmucha grisea* attacks the immature catkins of certain forest trees (Schaefer and Ahmad, 1987), but the level of damage caused is usually below economic injury levels. Elsewhere in Europe, the social biology of the maternal bug *Elasmucha grisea* has been investigated (Maschwitz and Gutmann, 1979, larval behavior; Melber et al., 1980, female adult egg-guarding behavior). Consideration might be given to studies of acanthosomatid populations in relation to present expectations of global warming. A northward spread of *Acanthosoma haemorrhoidale* in recent years in Britain has been reported (Southwood and Leston, 1959). Work on Pentatomidae (Aldrich, 1988a,b) has demonstrated the feasibility of population monitoring by pheromone trapping (Aldrich, 1988a,b).

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CHEMICAL MARKER FROM SILK OF *Yponomeuta cagnagellus*

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Abstract—Trail following in lepidopterous larvae is often attributed to chemical markers, but only a few clear-cut examples are found in the literature. In this paper evidence is presented for a chemical basis of the trail following behaviour of *Yponomeuta cagnagellus*. (Lepidoptera: Yponomeutidae) The marker is shown to be very persistent under laboratory conditions and is water soluble. Several possible secretory sites were investigated, and it is concluded that the marker is probably secreted together with the silk from the labial gland. Problems associated with the demonstration of trail markers in caterpillars are discussed.

Key Words—*Yponomeuta cagnagellus*, caterpillars, Lepidoptera, Yponomeutidae, trail following, chemical marker, trail pheromone, stability, pheromone secretory site.

INTRODUCTION

Trail following has been described for several species of lepidopterous larvae (see Fitzgerald and Peterson, 1988, for review). This behavior is thought to facilitate orientation and plays a role in larval aggregation (Long, 1955; Fitzgerald and Peterson 1983; Fitzgerald and Costa, 1986). In addition to these functions, it has been shown that in the genus *Malacosoma* "recruitment trails" are produced by successful foragers. Such trails can convey information about the quality of a distant food source and are preferred by colony members over exploratory trails (Fitzgerald, 1976; Fitzgerald and Peterson 1983; Peterson, 1986, 1988).

Although trail following in Lepidoptera is often attributed to chemical markers associated with the trail, only a few clear-cut demonstrations of a

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chemical basis for this behavior have been reported in the literature. Except for the results in *Malacosoma* (Fitzgerald and Gallagher, 1976; Fitzgerald and Edgerly, 1982; Fitzgerald and Costa, 1986; Peterson, 1988), only two other unequivocal cases have been found. The European birch tent moth *Eriogaster lanestris* has been shown to possess a trail marker (Weyh and Maschwitz, 1978). This species belongs to same family as *Malacosoma*, i.e., the Lasiocampidae. The other example is the saturniid *Hemileuca oliviae* (Capinera, 1980).

It can be concluded that trail pheromones are predominantly known from the Lasiocampidae. To broaden the knowledge of trail pheromone systems and the types of substances used in the Lepidoptera, trail following in *Yponomeuta cagnagellus* (Yponomeutidae) was investigated. Caterpillars of this species live gregariously in groups of 20–50 on the spindle tree *Euonymus europaeus* and produce conspicuous webs that may envelop the leaves of their food plant. During their development the larvae gradually extend the limits of this web to encompass new leaves. When the local food supply is exhausted, the colony often moves to a new feeding site, sometimes at a great distance from the original web. The larvae remain gregarious during this process as well as during their migration to the vegetation on the ground at the time of pupation (Hoebeke, 1987). A trail pheromone could facilitate this gregarious behavior.

It has been shown that *Yponomeuta* larvae are able to follow each other's trails (Roessingh, 1989). Tactile cues from the silk play a role in this behavior, while an additional chemical factor has been hypothesized (Roessingh *et al.*, 1988; Roessingh, 1989). In this paper experiments are described that establish the existence of such a trail marker. In addition the longevity of the trail under laboratory conditions was studied, as well as the effects of washing trails with water and organic solvents.

An important piece of evidence in the validation process of a trail pheromone is the localization of the secretory site (Howard et al., 1976). Several sites have been identified or proposed for caterpillars. In the genus *Malacosoma* a trail marker is produced on the ventral side of the abdomen (Fitzgerald and Edgerly, 1982; Fitzgerald and Costa, 1986; Peterson, 1988). Another possibility, suggested by Weyh and Maschwitz (1978), is that the marker originates in the labial glands (the silk glands) and is excreted together with the silk. This could also be the case for *Hemileuca oliviae* (Capinera, 1980). An alternative idea was formulated by Povel and Beckers (1982) in a paper describing the prothoracic organ, a gland on the ventral side of the prothoracic segment, that traditionally has been thought to have a defensive role. These authors stated that this gland does not seem to function in this manner in *Yponomeuta*, and they suggested that it might be the source of a trail pheromone. In addition to the glands already mentioned, *Yponomeuta* caterpillars also are equipped with prominent mandibular and pharyngeal glands as well as glands of Filippi (Berlese, 1909).

In an attempt to answer the question whether a trail pheromone is produced in *Yponomeuta*, extracts from glands and body parts were assayed for their ability to induce trail-following behavior. Additional information about the possible role of some of these structures was collected in experiments in which the release of chemicals was blocked and in tests with trails made out of chemicals wiped directly from the body surface of the caterpillars.

METHODS AND MATERIALS

Insects and Plants. Egg batches of *Yponomeuta cagnagellus* (Hübner) were collected in the field on *Euonymus europaeus* and kept at 6°C and 80% relative humidity until needed (cf. Bucher, 1959). Rearing was done in Petri dishes at 25°C and an 18:6 hr light–dark photoperiod on young *Euonymus* leaves taken from potted plants in the greenhouse. Plants were periodically cut back to induce development of new shoots with young leaves, essential for the survival of the first instar. The insects had continuous access to food and were used during the first four days of their fifth stadium.

Bioassay. All tests on natural trails were performed on filter paper Y mazes (Figure 1) modified from Fitzgerald and Edgerly (1979) and described in detail elsewhere (Roessingh et al., 1988; Roessingh, 1989). Paper proved to be

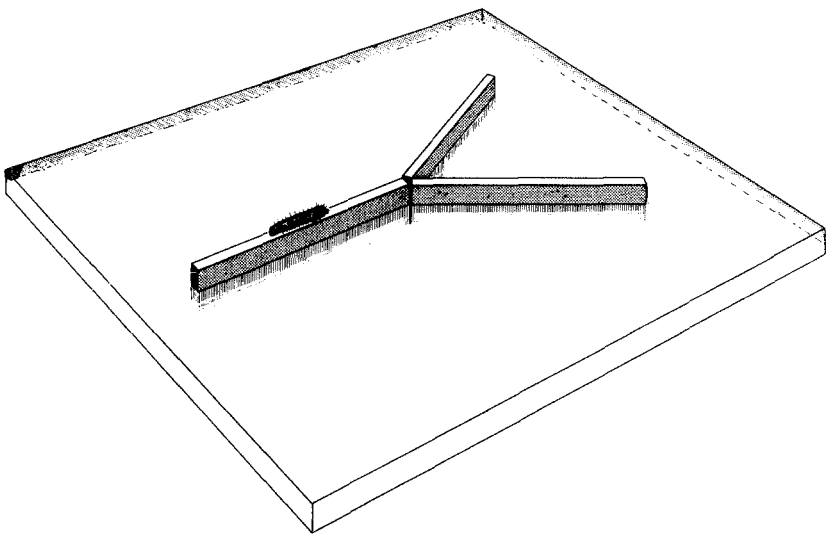


FIG. 1. Y maze used to determine the response of *Yponomeuta cagnagellus* caterpillars to extracts. The Y is made out of stainless-steel segments to prevent absorbtion of the extract.

unsuitable for testing extracts of parts of the caterpillar body, probably because the extract is absorbed in the paper and only small quantities of active material are present on the surface (Crump et al., 1987). To overcome this problem, stainless-steel segments, 50 × 2.5 mm wide, were used. Forty of these strips were placed in line and 25 caterpillars were allowed to lay a trail by crawling over this pathway. After the production of the trail, all sections were washed three times for 5 min in distilled water to inactivate the trail. The first 2 cm of 20 of these silk-covered metal segments were painted with 5 μl of caterpillar tissue extract, while the first 2 cm of the remaining 20 segments were painted with distilled water. One segment from each group was used to form an arm in the Y maze (Figure 1). Since *Y. cagnagellus* caterpillars are reluctant to enter silk-free substrates (Kalkowski, 1958; personal observations), the presence of the silk on both arms of the Y is important and facilitates the choice process.

Washing and Extraction Procedures. Washing of trails was done in Petri dishes in 100 ml of distilled water. The liquid was refreshed three times and gently shaken during the whole procedure.

Extracts were made from silk produced by caterpillars that had just moulted into their fifth stadium. When starved, these larvae display extensive locomotion and produce large amounts of silk, only minimally contaminated with fecal pellets. The few pellets that are produced can easily be removed. Quantities of silk with known weights were soaked in 1 ml of distilled water and stirred on a vortex for 5 min. The silk strands then were taken out and the remaining solution was centrifuged for 2 min at 10,000*g* to remove solids. The supernatant was subsequently concentrated at room temperature to yield 100 μl of silk extract.

For the gland and tissue extracts, fifth-stadium larvae were killed by immersion in dichloromethane for about 20 sec and dissected in distilled water. Labial glands, mandibular glands, and the prothoracic organ were removed. The last two segments of the abdomen and the head (containing the pharyngeal glands, the glands of Filippi, and the extreme distal parts of the labial and mandibular glands) were dissected also. Tissues from 40 or 50 larvae were immersed in 1 ml distilled water and disintegrated with a sonifier cell disrupter (model B-12, Branson Sonic Power Co., Soest, The Netherlands, equipped with a 3-mm microtip for use with small volumes). During this procedure, the tissue solution was kept chilled at 0°C. After homogenization, the solution was centrifuged for 2 min at 10,000*g*, and the supernatant was concentrated at room temperature to yield 100 μl of tissue extract.

Aging of Trails. The trails for the aging experiment were made by 25 caterpillars on filter paper strips. Aging of these trails was done in the lab in the dark in Petri dishes. No special measures were taken to control humidity or temperature. Aged trails were compared to fresh ones also made by 25 caterpillars. The test started within 45 min after production of the fresh trail.

Ablations, Wiping, and Sealing Experiments. The ablations were conducted with a HF microcautery device (Murphy Developments, Hilversum, The Netherlands) following the method described in detail elsewhere (Roessingh et al., 1988). Sealing of the prothoracic organ was done using wax with a low melting point and a laboratory-built temperature-controlled needle. Caterpillars were given 24 hr to recover from the treatment before they were used to produce trails. Before and after the trail production, the wax cover was checked to prevent contamination of the trail. Control trails were made by sham-treated larvae.

The test for an active surface residue from the prothoracic organ was done following the method used in the *Malacosoma* studies (Fitzgerald and Costa 1986; Peterson, 1988). Fifth instars were held firmly between two fingers, and the creased edge of a piece of filter paper was wiped over the exit of the prothoracic organ. The filter paper was then unfolded, and the linear trail that results was tested using a fresh caterpillar.

Statistical Analysis. The results of the Y-maze tests were analyzed using a G test for goodness of fit (Sokal and Rohlf, 1981). The numbers of larvae found on both arms of the Y were compared to the 1 : 1 ratio that is expected under the hypothesis that there is no difference in the number of larvae on the two arms of the Y maze. For the linear trails from the wiping experiment, a comparison was made with the results of a control experiment.

RESULTS

The existence of a trail pheromone can be demonstrated by determining behavioral responses to extracts from trails or trail silk. Washing trails with different solvents and testing the washed trails to see if the behavioral activity is lost allows the identification of solvents that removes the pheromone from the silk.

The effects of washing trails are summarized in Table 1. The effect of washing with hexane is small, no significant differences could be detected. Washing with H_2O, on the other hand, causes a large effect and almost all insects prefer untreated controls over H_2O-washed trails. Extended washing with dichloromethane (DCM) also influences trail attractiveness. The last two rows in Table 1 show a direct comparison between washing with H_2O and hexane, again indicating the strong effect of H_2O on the trail-following response.

More direct evidence for the existence of a trail pheromone is provided by the data in Figure 2. In these experiments, extracts of trail silk have been applied to a stainless-steel Y maze. Differential effects of the solvents on the structure of the silk (as might occur in the experiment described above) can now be excluded. From Figure 2 it is clear that extracts from 25 mg silk/ml or more

TABLE 1. EFFECTS OF DIFFERENT SOLVENTS ON FOLLOWING RESPONSE OF *Yponomeuta cagnagellus* CATERPILLARS

Solvent used on		Larvae on				
Branch A	Branch B	Branch A	Branch B	Wash time (min)	N	G[a]
None	Hexane	12	8	5	20	0.45^{NS}
None	Hexane	12	8	5	20	0.45^{NS}
None	H_2O	18	2	5	20	12.66**
None	H_2O	18	2	5	20	12.66**
None	H_2O	18	2	60	20	12.66**
DCM	H_2O	13	7	180	20	1.26^{NS}
DCM	H_2O	12	8	180	20	0.45^{NS}
Hexane	H_2O	17	3	5	20	9.18**
Hexane	H_2O	18	2	5	20	12.66**

[a] $^{NS}P > 0.05$, $^{**}P \leq 0.01$, G test for goodness to fit of 1 : 1 ratio.

are preferred over the plain solvent, indicating that a chemical marker is involved in the trail-following response of *Y. cagnagellus*. To demonstrate that this factor is characteristic for *Y. cagnagellus* and is not a general constituent of silk or brought about by contamination with fecal pellets, an additional series of assays was done. The results in Table 2 indicate that neither extracts from *M. neustria*

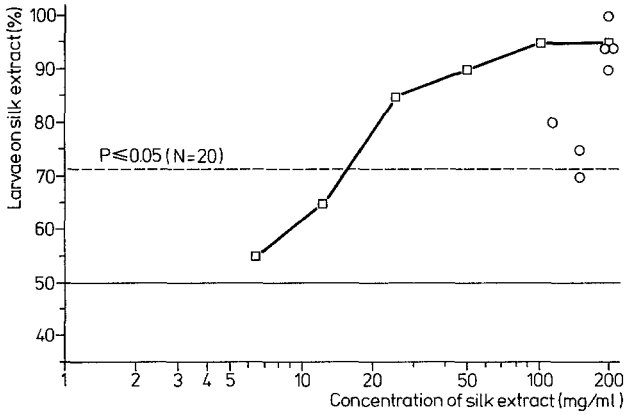


FIG. 2. Results of Y-maze tests with aqueous extracts from trail silk of *Y. cagnagellus*. The circles are results from tests with different batches of silk. The points on the curve are based on a dilution sequence of a single silk extract. The solid horizontal line at 50% indicates the expected outcome under the null hypothesis "no preference for silk extract." $N = 20$ for all data points.

TABLE 2. RESPONSE OF *Yponomeuta cagnagellus* LARVAE TO AQUEOUS SILK EXTRACTS, AQUEOUS EXTRACTS FROM FECAL PELLETS, AND H_2O CONTROL TREATMENTS

	Extract on:		Larvae on			
	Branch A (mg/ml)	Branch B	Branch A	Branch B	*N*	G[a]
Silk *M. neustria*	200	H_2O	8	12	20	0.45^{NS}
Silk *B. mori*	200	H_2O	7	13	20	1.26^{NS}
Silk *Y. cagnagellus*	200	H_2O	18	2	20	12.66**
Silk *Y. cagnagellus*	200	Fecal pellets 20 mg/ml	19	1	20	17.07**
Fecal pellets	16	H_2O	9	11	20	0.05^{NS}
Fecal pellets	20	H_2O	16	4	20	6.40*
Fecal pellets	20	H_2O	9	11	20	0.05^{NS}
Fecal pellets	67	H_2O	10	10	20	0.05^{NS}

[a] $^{NS}P > 0.05$, $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, G test for goodness of fit to 1 : 1 ratio.

or *B. mori* silk, nor fecal pellet extracts evoke a following response, but an water extract of *Y. cagnagellus* silk is again significantly preferred over the control.

To investigate the longevity of the marker, experiments were performed in which freshly produced trails were compared to trails aged in the laboratory for up to 200 days. Figure 3 gives an overview of the results. It can be concluded that even extensive aging of trails does not decrease their attractiveness compared to fresh trails. This indicates that the trail marker is nonvolatile and very persistent under laboratory conditions.

To determine the site of secretion of the marker, five possible sources have been examined: (1) the terminal part of the abdomen (Fitzgerald and Edgerly, 1982), (2) the labial gland (Weyh and Maschwitz, 1978; Capinera, 1980), (3) the prothoracic organ (Povel and Beckers, 1982), (4) the mandibular glands (Mudd and Corbet, 1984), and (5) the head, with the glands of Filippi and the pharyngeal glands. Tissue extracts from all of these sources were tested (Table 3). The only extract that is consistently active in the bioassay (although the larvae responded cautiously and showed extensive searching behavior) is that of the labial gland (the silk gland). Attempts to identify the exact region of the gland where the trail marker is produced were unsuccessful.

In other experiments to investigate whether or not the prothoracic organ produces the trail pheromone, this organ was sealed with wax. Trails made by these larvae were compared to those of sham-treated insects. No difference was

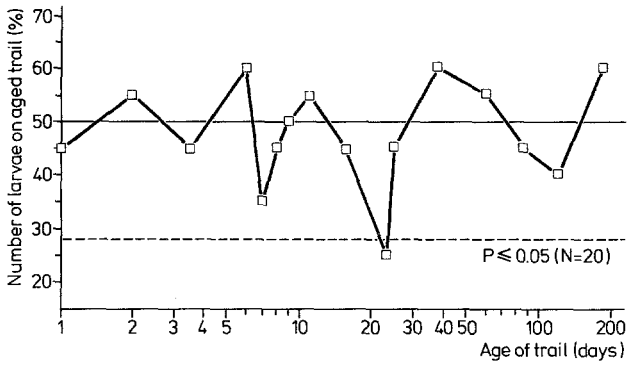


FIG. 3. Effect of aging on the preference for these trails over fresh controls. The solid horizontal line at 50% indicates the expected outcome under the null hypothesis "no difference between trails." $N = 20$ for all data points.

TABLE 3. RESPONSES OF *Y. cagnagellus* LARVAE TO EXTRACTS FROM GLANDS AND BODY TISSUE

	Larvae on:			
Extract	Extract	Control	N	G[a]
Head	35	25	60	1.36^{NS}
Abdomen	16	24	40	1.23^{NS}
Prothoracic organ	17	23	40	0.63^{NS}
Mandibular gland	17	23	40	0.63^{NS}
Labial gland	45	15	60	14.62**

[a] $^{NS}P > 0.05$, $*P \leq 0.05$, $**P \leq 0.01$, G test for goodness of fit to 1:1 ratio.

found between the trails of larvae with sealed and unsealed prothoracic organs (Table 4). Furthermore, trails produced by wiping with filter paper over the exit of the gland did not induce following behavior.

DISCUSSION

The main aim of this work was to demonstrate the existence of a trail pheromone associated with the silk trails of *Y. cagnagellus*. When the methods used to authenticate trail pheromones in lepidopterous larvae are reviewed, it appears that a standard procedure does not exist.

Several authors have suggested the presence of trail pheromones on the

TABLE 4. RESULTS OF Y-MAZE TESTS ON TRAILS MADE BY *Y. cagnagellus* LARVAE WITH SEALED PROTHORACIC ORGANS

	Larvae on trail made by larvae			
	With sealed organ	With unsealed organ	*N*	G
	13	7	20	1.26 ns
	12	8	20	0.45 ns
	7	13	20	1.26 ns
	11	9	20	0.05 ns
Total	43	37	80	0.31 ns

basis of the observation that caterpillars prefer conspecific trails over heterospecific trails (Fitzgerald and Edgerly, 1979, for *Archips*; Roessingh et al., 1988, for *Yponomeuta*; and Weyh and Maschwitz, 1982, for *Iphiclides*). While such results might provide good indications for the existence of trail markers, they do not yield unequivocal evidence. It has been demonstrated, for instance, that *Y. cagnagellus* can use tactile information from the trail (Roessingh, 1989), and therefore subtle cues from the silk structure cannot be excluded as a basis for the observed discrimination.

Other authors relied on washing trails with solvents to demonstrate trail markers (Gallagher and Lanier, 1977, for *Lymantria*; Masaki and Umeya, 1977, for *Hyphantria*; and Weyh and Maschwitz, 1982 for *Iphiclides*). Conclusions from these experiments are based on the implicit assumptions that: (1) the marker can be removed by the solvent used, and (2) the solvent does not change any other aspect of the trail that can be sensed by the caterpillars. Neither of these two assumptions can be justified. The results of Table 1 show the risk of relying on extraction without prior knowledge of the type of chemical involved. In the case of *Yponomeuta*, attempts to remove the trail marker with hexane are unsuccessful. Concerning the second point, it has been shown that silk can irreversibly change its shape and conformation in response to wetting (Gosline et al., 1986; Hepburn et al., 1979). For spider silk a 45% axial shrinkage has been reported together with a volume increase of about 15% (Work, 1977). Because of these effects, a change in silk structure rather than the removal of a chemical marker could explain the responses of the larvae.

Convincing evidence for the existence of chemical markers can be obtained by demonstrating trail following activity in response to solvent extracts of trails. This method was applied by Fitzgerald and Gallagher (1976) and Fitzgerald and Edgerly (1979) for *Malacosoma*, by Weyh and Maschwitz (1978) for *Eriogaster*, by Capinera (1980) for *Hemileuca*, and in this paper for *Yponomeuta*. Another good method is the direct transfer of the marker to filter paper by wip-

ing it over the caterpillar's body (Fitzgerald and Costa 1986; Peterson, 1988). In conclusion, it seems that the combined evidence of several experimental procedures may be needed to provide conclusive evidence for the existence of a trail pheromone.

Water extracts of trail silk from *Y. cagnagellus* showed activity in the trail-following bioassay described in the present paper. This indicates that a chemical marker is indeed associated with the silk. The marker is not a general constituent of silk, since extracts of silk from other species do not evoke following behavior. These findings contribute strong evidence that a pheromone is involved in the trail communication of *Y. cagnagellus.*

A plausible source of this marker is the prothoracic organ, as suggested by Povel and Beckers (1982). This gland terminates in a slitlike opening on a cone-shaped protrusion that is movable to some degree. Silk threads from the spinneret pass over this protrusion, and therefore a marker from this gland could be readily added to the newly spun silk. In view of the results presented here, it must be concluded that this hypothesis is no longer tenable. All approaches used to substantiate a role for this organ (blocking of the gland, assays of tissue extracts and surface residues) yielded negative results. In contrast, extracts from the labial glands did induce trail following behavior, and this is taken as evidence that the trail marker in *Yponomeuta* is secreted together with the silk.

In the experiments using extracts of labial gland tissue from 40 or 50 larvae, even this large amount of material yielded a trail that evoked only cautious reactions by the caterpillars and often induced extensive searching behavior. This result differs, for instance, from findings in termites and ants, which strongly respond to extracts from a single gland (Traniello, 1983; Jaffe and Howse, 1979). A possible explanation is that the trail marker in *Yponomeuta* is not stored in significant quantities in the gland, a situation comparable to that found in *Malacosoma* (Fitzgerald and Edgerly, 1982).

In gypsy moth larvae, *Lymantria dispar*, a water-soluble kairomone was found that elicits searching behavior in the braconid parasite *Cotesia* (=*Apanteles*) *melanoscela* (Weseloh, 1976, 1987). The labial gland was identified as the principal source of this substance (Weseloh, 1976, 1977). It has been argued that a kairomone also must possess some biological advantage for the emitter, otherwise production of the chemical would be selected against (Vinson, 1976). In *Lymantria*, larval migration is slowed down if silk trails are removed, and the presence of a chemical marker has been suggested (McManus and Smith, 1972; Gallagher and Lanier, 1977). Therefore it is conceivable that the primary function of the kairomone used by *Cotesia* is that of a trail marker, which is secreted together with the silk, like in *Yponomeuta.*

It has become clear that longevity of trail pheromones is correlated with their function (Bradshaw and Howse, 1984). Markers for orientation in the home range largely retain their information content over time and can be persistent.

In snails and slugs orientation cues have been described that persisted for days (Chelazzi *et al.*, 1985; Cook, 1979). For several termite species, it has been shown that extremely stable markers are produced that (under laboratory conditions) stay active over periods of months or years (Traniello, 1982; Runcie, 1987). Recruitment signals, on the other hand, carry information about food sources, and, when these become exhausted, the signal should disappear. Consequently these signals are, in general, volatile substances that are often only effective over periods of minutes (Bradshaw and Howse 1984; Hölldobler, 1977; Traniello, 1982; Runcie, 1987). More durable markers are used in situations with a stable food supply (Hölldobler, 1977). This might explain the relatively long lifetime of trail markers in phytophagous insects like leaf-cutting ants and caterpillars (Fitzgerald, 1976; Lewis et al., 1974; Peterson, 1988), as well as in *Yponomeuta.*

The extreme persistence of the *Yponomeuta* marker indicates that it must be largely nonvolatile. This is consistent with results from tests on *Y. cagnagellus* in a bioassay on trails shielded with nylon mesh. The mesh inhibited following behavior, although possible volatiles from the trail were still accessible to the larvae (Roessingh *et al.*, 1988). It was concluded that if a trail marker exists, it will be a contact cue. The observed persistence reported in this paper corroborates this conclusion.

These facts are not consistent with data published on *Y. evonymellus*, a near relative of *Y. cagnagellus*. Kalkowski (1958) reported a volatile trail marker for this species. The cause of this discrepancy is not clear. Given the close taxonomic relationship of the two species, it seems unlikely that these species use completely different trail pheromones. One possibility is that the marker is a multicomponent system consisting of a stable contact cue, and a short-lived rapidly evaporating volatile cue. A volatile marker disappearing within 30 min might have escaped attention in the present research on *Y. cagnagellus*. This explanation assumes that Kalkowski tested his trails within this time span, but the information on experimental procedures given does not allow conclusions on the likelihood of this interpretation.

From experiments in which *Y. cagnagellus* trails were treated with different types of chemicals, it is clear that the trail-following behavior of the larvae is not influenced by the apolar solvent hexane, while a polar solvent such as H_2O effectively eliminates the following response. This result indicates that the marker has a polar character and is water soluble.

A water-soluble marker seems to be of limited use under natural conditions and has led to some doubts about the adaptive value of some of these substances (Prokopy, 1975). On the other hand, water-soluble semiochemicals are not at all uncommon. Prokopy (1981) gives an extensive list of epideictic pheromones, and many of these have been found to be water soluble. The contact sex pheromone bound to the silk of the lycosid spider *Pardosa lapidicina* also

is readily inactivated by water (Dondale and Hegdekar, 1973), as is the kairomone present in the silk of the gypsy moth *Lymantria dispar* (Weseloh, 1976, 1977). Thus it may be concluded that many semiochemicals are washed away easily by rain or inactivated by dew, but this does not seem to prevent their successful use in nature (Averill and Prokopy, 1987). In some cases this may be related to the fact that only a limited activity period is needed for the substance to function. The oviposition-deterring pheromone of the leaf miner *Agromyza frontella*, for instance, is washed away in heavy rains, but a 24-hr activity period seems to suffice to give the larvae a competitive advantage. (Quiring and McNeil, 1984).

In the case of *Yponomeuta*, rain and dew might cause periodic removal of the information in the trail system, offsetting the long lifetime of the marker.

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INTERNEST AGGRESSION AND IDENTIFICATION OF POSSIBLE NESTMATE DISCRIMINATION PHEROMONES IN POLYGYNOUS ANT *Formica montana*

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Abstract—Polygynous ant species often monopolize patchily distributed habitats and tolerate neighboring conspecifics while aggressively attacking other ant species. We determined that internest aggression occurs in the polygynous ant, *Formica montana*. We report for the first time the identities of cuticular hydrocarbons of *F. montana* and present results of their possible role in nestmate recognition. Cuticular hydrocarbons contribute differentially to class discrimination, certain hydrocarbons being more class distinct.

Key Words—Nestmate discrimination, polygynous ants, cuticular hydrocarbons, multivariate analysis, aggression, prairie ants, class distinction, Hymenoptera, Formicidae.

INTRODUCTION

Most ant species discriminate between nestmates and conspecifics from other nests. Hamilton's (1964) theory of inclusive fitness predicts that the social organization of the colony, including aggressive behavior, is a function of the relatedness between individuals in the colony. In polygynous (i.e., having several functioning queens in a nest) ant species, however, the ability to distinguish nestmates from nonnestmates is often lost, presumably because of a reduced within-nest relatedness (Hölldobler and Wilson, 1977; Hölldobler and Michener, 1980; Breed and Bennett, 1987). Hölldobler and Wilson (1977) suggest

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that polygyny is a derived form of social insect organization (monogyny being the rule), brought about by ecological pressures, while the inability to discriminate between nests is an adaptive response that helps the species dominate and better utilize the habitat. Newly studied polygynous species, however, are aggressive to conspecifics from other nests (Rissing et al., 1986; Peeters, 1988; Crosland, 1989a,b), in which case the cause–effect relationship of polygyny needs reconsideration.

We studied the interaction behavior between colonies of *Formica montana* Emery in southern Wisconsin. This species is polygynous, polydomous (i.e., a colony may inhabit more than one nest), and tends to form large, highly aggregated populations in prairies and open-field habitats in southern Wisconsin (Henderson et al., 1989; Henderson, 1989). Our purpose was to determine if nestmate discrimination occurs in *F. montana*.

We also investigated the mechanism of nestmate recognition. Contacting ants generally show an immediate aggressive or passive response following a simple sweep of the antennae, indicating that surface chemicals are responsible for nestmate recognition (Fielde, 1903; Wilson, 1971). For some social insects, cuticular hydrocarbons (CHs) synthesized by cells located near the integument (Blomquist and Dillwith, 1985) are thought to serve as discrimination pheromones, since profile similarities (i.e., the relative likeness in proportion of major CHs shared by two organisms), are negatively correlated with aggression levels (Howard et al., 1980; Vander Meer and Wojcik, 1982; Obin, 1986; Bonavita-Cougourdan et al., 1987; Vander Meer, 1988; Espelie and Hermann, 1988). We verified that chemical cues on the body are used by *F. montana* to discriminate between friend and foe and present evidence that cuticular hydrocarbons may be involved in this recognition system.

METHODS AND MATERIALS

Three bioassays were performed on *F. montana* to measure aggression between nests and determine if nestmate–nonnestmate interactions differ. Two bioassays were conducted in the field and one in the laboratory. Field observations on *F. montana*, made between 1986 and 1989, added to the interaction behavior data presented below.

Bioassay 1. Conspecific *F. montana* from polydomous nests are not aggressive toward nestmates (Henderson et al., 1989; Henderson, 1989), but it is not known if *F. montana* defend their nest from nearby nests of conspecifics (those that are not polydomous). Aggression between foragers from two adjacent nests was examined at a location in Curtis Prairie (University of Wisconsin Arboretum, Madison, Wisconsin). The two nests were nearest neighbors (0.5 m separation), designated as Pond A nest and Pond B nest. Internest aggression

was determined through observations of contacts between individuals from both nests (approx. 20 hr total). In some instances we manipulated natural conditions (honey-tipped sticks were placed at various locations between the two nests) to enhance colony–colony interactions. An aggressive response between contacting individuals included biting, turning the gaster and spraying, pulling, and/or dragging. Field observations started April 1987, several days after a controlled burn of Curtis Prairie was carried out. The fire removed much of the underlying thatch that would otherwise make nest border interactions difficult to see.

Bioassay 2. We examined the aggression foragers displayed toward nestmates and nonnestmates along a foraging path at a location in Trout Creek (Department of Natural Resources land, 7 km south of Barneveld, Wisconsin) where seven *F. montana* nests circled a small (3 m tall) *Acer negundo* L. heavily infested with the aphid *Periphyllus negundinis* (Thomas). The aphids were tended by *F. montana* for their honeydew. Soil was dug and turned within a 1-m radius around the tree to provide an even textured pathway for better visibility of ant traffic (from the nest to the tree). In 1987 we established through observations that ants from all seven nests utilized the honeydew supply on the tree. In 1988 we again created the aboveground conditions for aggression bioassays.

Bioassays began July 6 and ended July 27, 1988. Ten foraging ants from each of the seven nests were collected by hand as they attempted to enter or exit their mounds, placed in scintillation vials, and killed by freezing on Dry Ice. After freezing, vials were uncapped and kept at ambient temperature for 10 min before testing began.

A test involved placing the recently killed ants in a small cluster along the exposed pathway and recording the number of foragers that showed aggressive responses (AR) and the number of total contacts (AR + passive responses, PR) for 3 min; all seven samples were presented one at a time in random order over the next hour. AR/(AR + PR) constituted the aggression index (AI). We used the foraging path of nest workers with the highest ant traffic, colony P41. An aggressive response was scored when a P41 forager bit, gaster sprayed, pulled and/or dragged a dead conspecific. Dead P41 ants served as a control group. Mann-Whitney U tests were performed to determine if there were significant differences in aggression indices (AIs) between the control group and each of the other six nests.

Bioassay 3. To determined the "completeness of recognition" between two nests, we looked at aggression between laboratory colonies of *F. montana*: We asked the question, how many ants are misidentified and accepted as nestmates? Two partial colonies were collected in June 1987, one from Trout Creek (colony TC) and one from a small field in Fitchburg, Wisconsin (colony F), and were placed in separate "ant farms" measuring 0.5 m × 0.3 m × 1.5 cm

(length, height, and width) with Plexiglas windows. Colonies were kept on a constant 10-hr light 14-hr dark cycle at 26 ± 2°C in an insect-rearing room. Ants were fed ad libitum a diet of cut-up mealworms (*Tenebrio molitor* L.), honey, and distilled water for nine months before the experiment was started.

Both colonies were producing brood in the laboratory by March 1988: colony F had several queens and was producing many workers; colony TC, however, was queenless and only male brood were present (a male alate was present and pupae were dissected and determined to be males).

We cooled colony TC by refrigeration (March 9, 1988), and all workers were marked (total = 536 workers) with a drop of typewriter correction fluid (Paper Mate, Gillete Co., Boston, Massachusetts) applied to the dorsum of the gaster. This marker does not modify the behavior of ants toward nestmates or nonnestmates (Henderson, personal observations). Colony F was not marked, and workers numbered well over 2000. After marking the ants, we waited 14 days and then connected the ant farms to a common feeding tray (supplied with mealworms, honey, and water) via Tygon tubing. Half-hour observations were taken daily thereafter until April 6, 1988.

Role of Cuticular Hydrocarbons (CHs). To determine the possible role cuticular hydrocarbons (CHs) play in discrimination, we conducted a field bioassay using ants washed with chemicals having different polarities. Chemical washes included distilled water, isopropyl alcohol, ether, hexane, and pentane (CH profiles are often determined from hexane and pentane extracts). We hypothesized that ants washed free of important recognition chemicals would be attacked significantly more than ants having the appropriate chemical cues. If CHs are involved, the less polar washes (e.g., pentane and hexane) should cause an increase in aggression toward nestmates and nonnestmates alike. For the bioassay, we again conditioned the surface soil around the box elder tree at Trout Creek (to make ant traffic more visible). We tested dead P99 (one of seven nests circling the box elder) and P41 ants against foraging P41 ants; both should be treated similarly if recognition cues are lost through washes. Ants were hand-picked as they entered or exited their nest and washed twice in one of the six solvents for 10 min (10 ants per Teflon-capped vial). Ants washed in distilled water were frozen on Dry Ice in order to kill them (while other solvents killed the ants immediately, ants revived from being submerged in water). P99 and P41 ants frozen on Dry Ice (and not washed) served as control groups. Trials were conducted as noted in bioassay 2; however, the observation time was increased from 3 min to 10 min to increase the number of contacts used to calculate the aggression index (AI). In this bioassay, presentation of a treatment was completely randomized for three complete tests (for both P99 and P41) of a wash; on any one day six treatments were presented (one at a time) to a foraging P41 group within a 2-hr period. Testing was conducted between July 6 and 12, 1989, a time when brood was present in the nests. Significant differ-

ences between ant colonies, washes, and in the ant colony–wash interaction were determined using a two-way ANOVA and the statistical software package, Minitab (Minitab Inc.). AI proportions were arcsine transformed to stabilize the variance.

Determination of CH Profile. Sixteen *F. montana* samples were collected by hand from two distant nests (15 foragers/sample; eight samples per nest) at Curtis Prairie in August 1987. All ants collected were kept separate, brought back to the laboratory, anesthetized within 24 hr with CO_2 gas, and placed in Teflon-capped vials containing 5 ml of *n*-pentane. Samples were washed for 5 min in 5 ml of *n*-pentane three times, and the resulting supernatants pipetted into small Teflon-capped vials and concentrated under a stream of purified nitrogen to a volume of 0.5 ml. Initially, the washed extracts were passed through a small column (made from a Pasteur pipet packed with 4 cm of silica gel) and eluted with 5 ml of *n*-pentane. The gas chromatogram (GC) profile did not change after this cleanup method, so this step was eliminated.

CH extracts were analyzed by capillary GC on a 15-m × 0.25-mm (ID) DB-5 column (J&W capillary; Alltech Associates, Deerfield, Illinois). The temperature programming conditions were 150°C for 0.5 min, then 8° min to 250°C with a 15-min hold. Injector and detector temperatures were 275°C. Flow rate was 1.22 cc (He) per minute. Mass spectrometry (MS) was performed on a Finnigan 4000 GC-MS equipped with a 15-m × 0.25-mm (ID) DB-5 column; energy = 70 eV, 1.55 kV. Comparison with authentic standards on the capillary GC and mass spectrometry were used to identify alkanes. Alkenes were identified by mass spectrometry only, as standards were not available at the time.

The major chromatogram peaks generated by individual CHs were relativized for each sample first by subtracting from the total proportional makeup (total AREA%) all chromatogram retention times outside of those between 6.13 min and 13.94 min (over 80% of the CHs found on *F. montana* occurred between these retention times), and then dividing the AREA% given for each CH by the total AREA% of the CH profile under consideration. This gave a relative proportion for each CH that could then be quantitatively compared between samples.

Determination of Class-Distinct CHs. We determined which CHs were class distinct by comparing peak (generated by the chromatogram) proportional contributions among the 16 samples from the two nests. Although CHs that are highly variable in their proportional contribution may be important for species recognition, for class distinctions (i.e., kin-group recognition, see Crozier, 1988), CH profiles should be similar within a nest and distinct between nests. Class distinction was determined by ranking the number of CH overlaps: the number of times the proportional contribution of a CH from one set of nest samples overlapped with the range of proportional contribution from the other set of nest samples.

Multivariate Analysis of CH Profiles. We examined the relationship of CHs among *F. montana* nests using the software package PC-ORD (Holcomb Research Institute, Butler University, Indianapolis, Indiana), a menu-driven and hierarchical system for multivariate analysis of ecological data (McCune, 1987). For the analysis, *Formica montana* foragers (and in one case two queens alone) were collected by hand from eight colonies at three sites in southern Wisconsin: Curtis Prairie, Trout Creek, and Turville-Olin Park (Madison, Wisconsin). Samples from the same nest were collected from two nests at Curtis Prairie, one from Turville-Olin Park, and two from Trout Creek (total = 13 samples from eight nests). We analyzed the CH profile and relativized CHs under consideration following the protocol given above. We chose different groups and combinations of CHs (3–10 CH group combinations, based on the previously determined overlap rankings and determined their relationship to the 13 nests using cluster analysis (CL; a classification technique) and principal component analysis (PCA; an ordination technique).

Our purpose was to determine if CHs showing a minimal number of CH overlaps show grouping patterns that indicate kin-group recognition.

RESULTS

Aggression between *F. montana* foragers from different colonies was observed often in the field. Fighting occurred near food resources (honeydew) and near nests. Fighting was always sporadic and never en masse. Occasionally, an aggressor dragged her opponent toward a nest. At other times, victims were observed "pinioned" by several workers each holding a leg or antenna.

Bioassay 1. Forty colony–colony episodes of aggression were observed between members of Pond A nest and Pond B nest; despite their spatial closeness, nearest-neighbor aggression was evident. Ants attacked foragers from the opposite nest more frequently when honey sticks were placed between the nests; when placed at the appropriate spot (which was not at the midpoint between nests, but more towards one nest than the other), fighting increased such that on one day seven fights were occurring simultaneously. When honey sticks were not in place, it was evident that foragers avoided the space between nests; foraging paths to food resources did not normally intersect. Learning of neighboring territories may influence foraging direction.

Fighting often included several workers "pinioning" an opponent. Interestingly, several times an opponent was observed being dragged into the aggressor's own territory. Once there, other nestmates then joined in on the attack. A cartwheel effect could be created by placing ants that were pinioning an opponent into the victim's own territory. Nestmates of the victim would grasp the legs of the aggressors (those pinning down the single adversary).

Bioassay 2. Ants responded to dead conspecifics from some nests by attacking them more often than the control (nest P41; Table 1). Behavioral cues are not necessary for *F. montana* to respond differently to nestmates and nonnestmates. Dead P41 ants were attacked by foraging nestmates occasionally; they were also sometimes licked. Three nests showed nonsignificant differences in their aggression index (AI) when compared with the control; they were the closest nests to P41 and may represent a polydomous colony that includes nest P41. The three remaining nests had much higher AI values than the control. P41 foragers tended to spend more time investigating dead ants from the latter three nests.

Bioassay 3. Every member ($N = 536$) of the Trout Creek (TC) colony was attacked by the Fitchburg (F) colony and killed. The fighting between these colonies is less dramatic than between colonies of some ants. For example, fighting behavior between *Tetramorium caespitum* (Linne) colonies involves several hundred ants simultaneously engaged in battle (McCook, 1909; Henderson, personal observations). At first, TC ants appeared to be of little concern to the F colony. While fights occurred sporadically, some TC ants were seemingly accepted by the F colony, sometimes seen moving about freely in the F colony's ant farm. However, two days into the bioassay (March 25, 1988) only about 200 TC ants were alive; they remained stationary and aggregated in a corner of their ant farm. F ants constantly patrolled the TC aggregation and, during most observation periods, pulled one or more TC ants from the cluster. Those pulled from the cluster were then mass attacked and pinioned. TC ants behaved timidly in comparison to the more aggressive F ants and were not observed to initiate a single fight. Two weeks later (April 6, 1988), every TC

TABLE 1. AGGRESSION INDEX (AI) MEANS FOR SEVEN *F. montana* NEST FORAGER GROUPS KILLED AND PRESENTED TO ACTIVELY FORAGING NEST GROUP (P41) AND TEST OF INDEPENDENCE OF AIS USING MANN-WHITNEY TEST COMPARING AIS FOR SIX NESTS WITH AI OF CONTROL NEST (P41)[a]

Nest	AI	SD	Significance level
P41 (control)	0.082	0.083	
P1	0.117	0.126	0.83
P52	0.233	0.252	0.51
P45	0.333	0.167	0.13
P34	0.392	0.101	0.08
P13	0.702	0.287	0.08
P99	0.557	0.051	0.08

[a] SD = standard deviation of AI based on three trials.

member had been eliminated; there was no integration of the TC ants into the F colony.

Washes. The difference in P41 aggression (measured by the AI) toward dead P41 and P99 ants was dependent on the polarity of a wash and independent of the ants' nest origin (Figure 1). As polarity of the wash went down, the AI went up significantly (two-way ANOVA, $F_{5,24} = 6.92$, $P < 0.001$). The biggest difference in response to a P41 versus a P99 treatment was in the control; this difference was significant [Fisher's least significant difference (LSD) method, $P < 0.05$]. Thus, in all treatments except for the control, P41 foragers reacted to dead P41 washed free of CHs (or at least partially free) in the same way that they reacted to nonnestmates, in this case, P99 ants. Water-washed ants showed little difference in AI when compared with the controls. Interestingly, however, while the water-washed P41 ants had a slight increase in the AI (from its control group), the water-washed P99 ants had a reduced AI. Since cuticular hydrocarbons (CHs) are washed most efficiently from the cuticle by the nonpolar washes, the results suggest that CHs may be discrimination pheromones for *F. montana*.

CH Profile and Class Distinction. The CH makeup between colonies was qualitatively similar but quantitatively different (Table 2). All identified alkanes were unbranched. All identified alkenes were also linear, but further analysis is

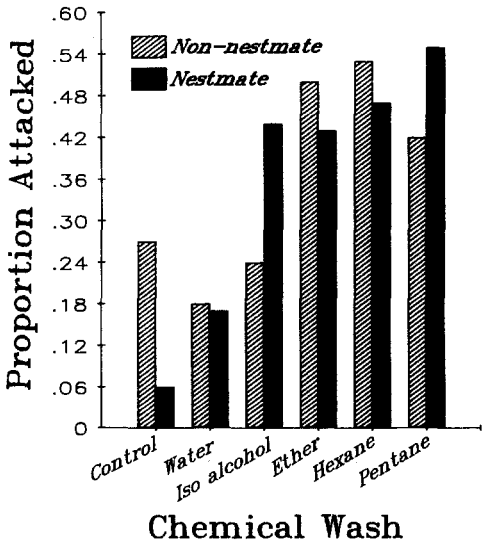


FIG. 1. Response of foraging P41 ants on contact with dead nestmates and nonnestmates washed with chemicals of different polarities. From left to right washes decrease in polarity. Hexane and pentane are equivalent in polarity. Control = unwashed ants, iso = isopropyl.

TABLE 2. MAJOR CUTICULAR HYDROCARBONS OF *Formica montana*[a]

Hydrocarbon	Carbon number	Retention time (min)	Proportion present ± SD		Overlaps
			Nest A	Nest B	
n-Heneicosane	21	6.13	0.030 ± 0.004	0.018 ± 0.003	0
n-Docosane	22	7.16	0.004 ± 0.003	0.002 ± 0.002	4
n-Tricosene	23	7.89	0.045 ± 0.008	0.019 ± 0.007	0
n-Tricosane	23	8.18	0.063 ± 0.006	0.055 ± 0.010	5
n-Tetracosene	24	9.19	0.008 ± 0.005	0.021 ± 0.011	2
n-Tetracosane	24	9.86	0.014 ± 0.013	0.032 ± 0.007	1
n-Pentacosene	25	9.91	0.126 ± 0.017	0.090 ± 0.021	1
n-Pentacosane	25	10.18	0.100 ± 0.012	0.159 ± 0.060	1
n-Hexacosene	26	10.88	0.020 ± 0.002	0.024 ± 0.004	2
n-Hexacosane	26	11.14	0.015 ± 0.002	0.029 ± 0.012	1
n-Heptacosene	27	11.84	0.383 ± 0.030	0.345 ± 0.104	5
n-Heptacosane	27	12.07	0.056 ± 0.003	0.096 ± 0.041	0
n-Octacosene	28	12.74	0.003 ± 0.003	0.003 ± 0.004	4
n-Octacosane	28	12.98	0.002 ± 0.002	0.006 ± 0.006	5
n-Nonacosene	29	13.68	0.016 ± 0.004	0.015 ± 0.004	4
n-Nonacosane	29	13.94	0.010 ± 0.002	0.024 ± 0.015	1

[a] Comparison of cuticular hydrocarbon (CH) makeup between foragers from two distant nests (A and B). Proportion present is the mean of eight samples. Number of overlaps is the number of times the proportional contribution of a CH from eight samples taken from nest A overlap with the CH range from eight samples collected from nest B. SD = standard deviation.

needed to determine the position of the double bonds. Overlaps of CH proportional contributions was 0 (i.e., showed complete class distinction) for *n*-heneicosane, *n*-tricosene, and *n*-heptacosane only, all other CH comparisons showed some overlap. *n*-Heptacosene is the major contributor to the *F. montana* CH profile; however, it showed little class distinction and probably does not play a role in nestmate discrimination.

Multivariate Analysis. Both PCA and CL gave little information for hypothesis generation. Very few samples collected from the same nest and very few nests collected from the same locale paired in any systematic way. The number of CHs added to the analysis did not improve the classification or ordination of the samples.

DISCUSSION

Pamilo et al. (1978) have shown that relatedness among individuals in multiple-queen nests of mound-building *Formica* is much reduced from that of monogynous colonies and, clearly, relatedness is a key factor in nestmate rec-

ognition via kin-level selection hypotheses. However, polygynous *F. montana* recognize nestmates from nonnestmates, attacking dead conspecifics from neighboring nests more frequently than dead nestmates. Similar findings were discovered in studies on intraspecific aggression in the polygynous ant *Rhytidoponera confusa* in Australia (Crosland, 1989a). One possible reason nestmate recognition occurs in polygynous ant species is that colony relatedness remains relatively high. Recent evidence on colony budding in polygynous *Solenopsis invicta* Buren indicates that newly inseminated queens do not distribute themselves randomly but tend to establish new nests with females that were nestmates previously (Vargo and Porter, 1989).

That behavioral cues are not responsible for aggression supports the hypothesis that chemical cues are used in nestmate recognition. Our results also support the hypothesis that cuticular hydrocarbons (CHs) are used in nestmate recognition by prairie ants. However, *F. montana* have class distinctive CHs, suggesting that, while the entire CH profile may be important in species identification, class discrimination is more likely based on quantitative differences in a very few CHs. Rather than recognition through a blend of all odors (Crozier and Dix 1979, Hölldobler and Michener 1980), it appears that prairie ants may key in on specific CH groups. In a similar manner, dimethylalkanes characterize the chemical signature of different *Camponotus vagus* colonies (Bonavita-Cougourdan et al., 1987).

Multivariate analysis, however, did not support the hypothesis that certain CHs are used in nestmate recognition. Increasing the number of hydrocarbons analyzed in a stepwise fashion from three to 10 did not change the CL or PCA results in any predictable way. However, we cannot assume that our multivariate analyses mimic the integration of recognition cues used by the ants themselves.

Polygynous *F. montana* can discriminate between nestmates and nonnestmates. However, while a large number of contacts did not result in aggression in the field, the recognition system was nonyielding in the laboratory, and all nonnestmates were eventually killed. That many contacts are nonaggressive ones may allow colonies of conspecifics to coexist more closely in a habitat than could colonies of mixed ant species. *F. montana* do not react in the same manner when confronted by *F. obscuripes* in the laboratory. In a similar experiment to bioassay 3, an *F. montana* colony was connected to a feeding tray shared by *F. obscuripes*. Both colonies engaged the other in immediate battle, and in one day the fighting was over and all *F. obscuripes* ($N = 150$) were killed.

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SURFACE LIPIDS OF SOCIAL WASP *Polistes metricus* SAY AND ITS NEST AND NEST PEDICEL AND THEIR RELATION TO NESTMATE RECOGNITION

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Abstract—In eight replicate laboratory tests where *Polistes metricus* adults were allowed to choose between their own nest, a second nest, and neither nest, they selected their own nest 66% of the observed time. When the surface hydrocarbons had been extracted from the nests, the wasps chose their own nest only 8% of the time, but after the hydrocarbons were reapplied to the surface of the respective nests, they selected their own nest 47% of the time. These changes are significant. The cuticular lipids were analyzed from individual *P. metricus* adult females collected from 13 colonies. Surface lipids were recovered from the paper and pedicels of their nests. Eighteen hydrocarbons were identified in these lipid fractions. The major components of the wasp cuticular lipids were *n*-heptacosane, *n*-nonacosane, methylhentriacontane, and methyltritriacontane. Factor analysis revealed that extracts of pedicels are all similar in composition, while cuticle and paper extracts vary, sometimes similarly according to colony identity.

Key Words—*Polistes metricus*, Hymenoptera, Vespidae, social wasps, cuticular hydrocarbons, nestmate recognition, nest pedicel.

INTRODUCTION

Cuticular hydrocarbons provide a hydrophobic barrier that protects insects from desiccation (Blomquist and Dillwith, 1985; Hadley, 1985; Lockey, 1988). For several insects, surface hydrocarbons also have been shown to serve as sex pheromones or recognition factors and, in other cases, to provide thermal insulation and protection from predators (Howard and Blomquist, 1982; Hadley, 1985).

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A wide variety of insect hydrocarbons have been identified, and the cuticular hydrocarbon complement of a particular insect usually has many components. These hydrocarbons are either *n*-alkanes, alkenes, or methyl-branched, and they may have a wide range in chain length. The branched hydrocarbons usually have one, two, or three methyl branches, each of which can be located on several different carbons in the backbone of the chain (Jackson and Blomquist, 1976; Nelson, 1978; Lockey, 1985; Blomquist et al., 1987). Insect cuticular hydrocarbon fractions, therefore, may be very complex structurally, and there is a high potential for these components to be utilized for various informational roles.

Kin recognition factors are thought to be chemicals that are found in the cuticle and that can be recognized by other members of the colony (Wilson, 1971; Hölldobler and Michener, 1980; Carlin and Hölldobler, 1983; Fletcher and Michener, 1987). Several studies of kin recognition in bees, ants, social wasps, and termites have implicated cuticular hydrocarbons as the components involved in this recognition process (Howard et al., 1982; Post and Jeanne, 1984; Brill et al., 1985; Obin, 1986; Bonavita-Cougourdan et al., 1987; Mintzer et al., 1987; Blum, 1987; Vander Meer et al., 1989a). This possibility is supported by the fact that several species of insects that cohabit with ants or termites have been shown to have cuticular hydrocarbon patterns that are very similar to those of the specific ants or termites and are thought to be utilizing chemical mimicry to overcome normal nestmate recognition (Howard et al., 1980; Vander Meer and Wojcik, 1982; Espelie and Hermann, 1988; Vander Meer et al., 1989b). The nest material of ants has been shown to have an odor that affects the ants and seems to play a role in kin recognition (Hangartner et al., 1970; Hubbard, 1974). Components of Dufour's gland secretion (used to line brood cells) covary among nestmates in at least one social bee (Smith and Wenzel, 1988).

The nest has been clearly shown to be critical in establishing nestmate recognition in several genera of social wasps (Shellman and Gamboa, 1982; Pfennig et al., 1983; Ryan et al., 1985; Gamboa et al., 1986; Venkataraman et al., 1988). Having demonstrated that exposure to the nest carton is necessary and sufficient for later kin recognition, Gamboa et al. (1986) conclude that the cues are learned from the nest material, rather than from nestmates, in the first few hours of adulthood. In most species the nest carton itself is reinforced with various secretions (Wenzel, 1990), especially in the region of the pedicel (Jeanne, 1970, 1975; Turillazzi and Ugolini, 1979; Kojima, 1982, 1983), but it is not yet known which of these are relevant in the context of kin recognition.

In the present paper we characterize the hydrocarbons extracted from the cuticle of *Polistes metricus* adults, the paper of their nests, and the pedicel of their nests. We also examine the role that these hydrocarbons may play in nestmate recognition.

METHODS AND MATERIALS

Bioassay. Nests of *P. metricus* were collected from various locations in and around Athens, Georgia, in July 1989. The nests and wasps were brought to the laboratory and utilized in the bioassay within 24 hr of capture. Two nests of approximately equal size were employed in each bioassay. The pedicels were severed from the nests, and all larvae were removed. The nests were attached to the ceiling of a Plexiglas chamber (36 × 60 × 45 cm), and all the adult females that had been captured with one of the nests (two to eight individuals) were released into the chamber. After 30 min, the number of wasps on their own nest, the other nest, or neither nest was recorded and wasps were gently removed from both nests. Six additional recordings were made at 20-min intervals. The animals were removed, and the wasps from the other nest were then introduced for a reciprocal test. The nests were then removed from the chamber, immersed in hexane (200 ml) for 2 min, placed in a hood and dried with the aid of a hair dryer, and returned to the ceiling of the test chamber. After 30 min, the location of the wasps was recorded; a series of seven readings of wasp location were taken at 20-min intervals as described above. The reciprocal test was then performed with the other wasps. The nests were again removed from the chamber. The hexane extracts of the nests were concentrated to dryness with a rotary evaporator with the bath kept at 35°C. The extracts were resuspended in 0.5 ml redistilled hexane and uniformly applied in 5-μl aliquots with a Hamilton syringe to the outer surface of the nest from which they had been recovered. The nests were returned to the chamber and, after 30 min, the location of the wasps was recorded seven times at 20-min intervals as above, and the reciprocal test was performed.

Extractions. Wasps and nests were collected from 10 widely separated sites in Clarke and Jackson counties, Georgia, in June and July 1989. Wasps were frozen at −20°C and then extracted in redistilled hexane at room temperature for 60 sec (Blomquist et al., 1987). Pedicels were severed from the nest and extracted in hexane for 2 min at room temperature. Surface lipids were recovered from the nest paper by directing a stream of hexane at the outer surface of the nest and collecting the hexane in a beaker beneath the nest.

Chemical Analysis. The hexane extracts were concentrated to dryness under a stream of nitrogen. The samples were resuspended in hexane (50–500 μl) and aliquots (usually 0.1–1%) were analyzed by combined gas chromatography–mass spectrometry. A Hewlett Packard 5890A gas chromatograph was equipped with a 12-m or a 25-m cross-linked methyl silicone capillary column with helium as the carrier gas. The oven temperature was kept at 55°C for 3 min after injection (splitless), and then the temperature was increased at a rate of 25°/min to a final temperature of 305°C. The column was connected to a Hewlett Packard 5970 mass selective detector and mass spectra were recorded at 1.2-

sec intervals at 70 eV. Data were analyzed with a Hewlett Packard Chem Station containing an NBS Library of 40,000 mass spectra, and individual components were characterized by their mass spectra and determination of their equivalent chain lengths (Nelson, 1978; Blomquist et al., 1987). Quantitation was based upon peak areas of total ion chromatograms of standard hydrocarbons (Sigma Chemical Co.) (Espelie and Bernays, 1989).

Statistical Analysis. GC-MS results were analyzed using the SPSS statistical software package on an IBM-XT computer using arcsine transformations of the values for percent composition. Factor analysis was used to determine the major components of variation among the extracts. Separate analyses examined the complete data set and a reduced data set that omitted the pedicel extracts from consideration.

RESULTS

Tests of whether wasps were more or less likely to sit upon their own nest versus on the alien nest or anywhere else in the enclosure show that the extractable hydrocarbons are indeed important in determining wasps' recognition of, or preference for, their own nest carton. In total, wasps were on their own nest 66% of the recorded times prior to extraction of the carton hydrocarbons, 8% after extraction, and 47% after restoration of the hydrocarbons. Table 1 shows that for eight nests and a total of 39 wasps, all eight replicates showed fewer counts of wasps on their own nest after extraction than before, and the results for each test are highly significant. When the extracts were applied to the nest, restoring the original hydrocarbons, there were significantly more wasps upon their own nest in seven cases, while for one colony (number 19) there was no significant change from the previous distribution in the enclosure (Table 1).

The cuticular hydrocarbons of *P. metricus* adults were found by GC-MS analysis to be a mixture of *n*-alkanes, methylalkanes, and dimethylalkanes. The major *n*-alkanes were *n*-heptacosane and *n*-nonacosane, while the dominant methyl-branched hydrocarbons were a series of positional isomers of methylhentriacontane and methyltritriacontane (Table 2). The same hydrocarbons were found on the surfaces of the paper and pedicels of the wasps' nests, although there were variations in the relative amounts of the different hydrocarbons (Table 2). In several cases the hydrocarbon pattern of an individual wasp was very similar to the pattern obtained from the surface of that wasp's nest (Figure 1A and B; D and E). The cuticular hydrocarbons recovered from the wasps from colonies 10 and 13 have average chain lengths that differ from one another, but are quite similar to those recovered from their respective nest paper (Table 2). Extracts of wasps from these two nests are shown because they are examples of wasps that have relatively high (nest 10) and low (nest 13) amounts of C_{33}

TABLE 1. NEST SELECTION PREFERENCE OF *Polistes metricus* WASPS IN CHAMBER CONTAINING OWN NEST AND ANOTHER NEST

	Nest selection[a]					
	Own	Other	None	Own	Other	None
	Nest 14[b] (5)[c]			Nest 15 (5)		
Before extraction	21	0	14	25	2	8
After extraction	4	4	27	6	1	28
After reapplication	15	2	18	17	0	18
	Nest 16 (2)			Nest 17 (8)		
Before extraction	9	0	5	36	0	20
After extraction	0	1	13	2	13	41
After reapplication	2	2	10	22	26	8
	Nest 18 (6)			Nest 19 (2)		
Before extraction	24	4	14	8	0	6
After extraction	1	10	31	1	0	13
After reapplication	16	4	22	8	0	6
	Nest 20 (7)			Nest 21 (4)		
Before extraction	36	2	11	21	1	6
After extraction	4	5	40	3	2	23
After reapplication	31	4	14	16	3	9

Significance of tests[d]			
Preference for own nest[e]	$P < 0.001$	$P < 0.05$	NS
Fewer after extraction than before	8	0	0
More after restoration than after extraction	6	1	1

[a] The location of the wasps was recorded seven times at 20-min intervals. The wasps were on either their own nest, the other nest, or no nest (none).
[b] Nests listed in the same row were paired with each other in the chamber in separate tests (i.e., 14 and 15).
[c] The number of wasps tested with each nest is listed in parentheses.
[d] $N = 8$ nests; $N = 39$ wasps.
[e] All tests are chi-square but for nest 19 in which exact binomial probabilities were calculated due to small expected values.

and C_{35} methylalkanes and dimethylalkanes. The proportions of long-chain, branched hydrocarbons comprised the most obvious difference between the extracts of the wasps from the various colonies. The hydrocarbon patterns obtained from the surface of the pedicels were all very similar to one another,

TABLE 2. HYDROCARBON COMPOSITION (%) OF CUTICULAR LIPIDS OF WASPS AND EXTRACTS OF SURFACE OF NEST PAPER AND SURFACE OF NEST PEDICEL FROM TWO *Polistes metricus* NESTS

			Nest 10			Nest 13		
Peak[a]	ECL[b]	Component[c]	Wasp	Paper	Pedicel	Wasp	Paper	Pedicel
1	23.0	*n*-Tricosane	0.13	0.54	0.14	—	2.56	—
2	25.0	*n*-Pentacosane	3.01	4.69	1.46	5.54	5.85	2.96
3	26.0	*n*-Hexacosane	0.49	0.77	0.22	0.83	0.74	0.38
4	27.0	*n*-Heptacosane	12.54	14.48	3.60	29.39	21.42	6.44
5	27.3	9-,11- and 13-Methylheptacosane	2.97	2.16	4.83	3.55	2.05	3.48
6	27.7	3-Methylheptacosane	0.26	0.39	0.23	0.96	1.15	0.33
7	28.0	*n*-Octacosane	0.68	0.77	0.44	1.00	0.23	0.54
8	29.0	*n*-Nonacosane	14.49	7.75	2.06	12.45	8.37	2.33
9	29.3	11-,13- and 15-Methylnonacosane	8.36	8.29	12.24	15.44	7.23	13.82
10	30.0	*n*-Triacontane	0.63	0.85	1.15	0.80	0.83	0.92
11	31.0	*n*-Hentriacontane	1.24	1.36	0.35	0.74	1.63	0.34
12	31.3	11-,13- and 15-Methylhentriacontane	14.26	15.70	20.83	17.89	13.79	22.37
13	31.6	11,15- and 13,17-Dimethylhentriacontane	1.09	1.84	2.57	1.47	0.73	6.30
14	32.0	*n*-Dotriacontane	0.34	0.29	0.53	—	—	0.51
15	33.3	11-,13-,15- and 17-Methyltritriacontane	14.48	10.75	11.57	5.33	7.28	11.55
16	33.6	13,17- and 15,19-Dimethyltritriacontane	7.63	5.95	6.87	2.01	2.81	10.77
17	35.3	13-,15- and 17-Methylpentatriacontane	5.61	5.88	5.53	0.53	2.73	4.43
18	35.6	11,15- and 13,17-Dimethylpentatriacontane	7.90	9.74	7.54	1.49	3.20	9.88
Average chain length[d]			30.6	30.5	31.1	28.9	29.2	31.1

[a] Peak numbers correspond to those in Figure 1.
[b] Equivalent chain length.
[c] Components are only listed if their presence was confirmed by mass spectral analysis.
[d] Based on the number of carbons in the backbone of identified components.

but not necessarily similar to the patterns obtained from the respective wasps and nest paper (Figure 1C and F). The average chain length of the hydrocarbons extracted from the pedicels of the two nests in Table 2 were both 31.1.

The results of the GC-MS analyses of the extracts of the wasps, nest paper, and pedicels of the 13 nests were examined by factor analysis in an effort to identify similarities between the cuticular hydrocarbons of individual wasps and the hydrocarbons recovered from their respective nests. The first two factors explained 57.3% of the variation in the complete data set. The factor loadings for each of these components is given in Table 3. Factor I is primarily a contrast between shorter-chain-length (C_{23}–C_{29}) *n*-alkanes and longer-chain-length (C_{29}–C_{35}) branched alkanes. Factor II is not easily interpretable.

A plot of factor I versus factor II is shown in Figure 2. The pedicel extracts

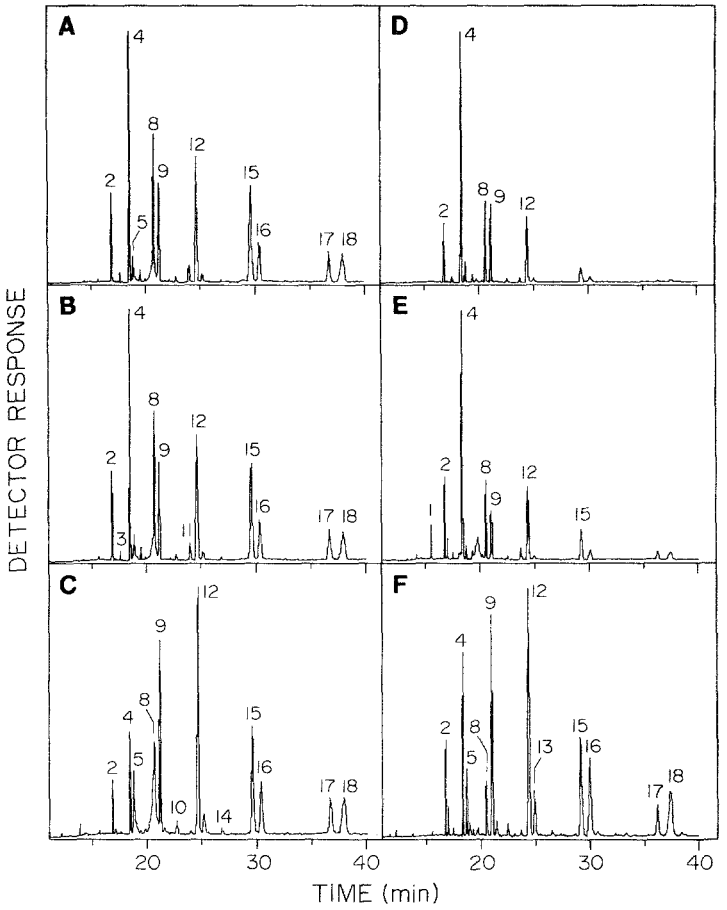


FIG. 1. Total ion chromatograms of the hexane extracts of *Polistes metricus* adult (A), nest paper (B), and nest pedicel (C) from nest 10; and adult (D), nest paper (E), and nest pedicel (F) from nest 13. Numbered components are identified in Table 2.

are clustered in a region that is isolated from the extracts of wasps and nest paper. Several of the wasp extracts plotted in the same region as the extracts of their respective nest paper (nests 1, 4, 5, and 10). In some cases, the extracts of two or three wasps from the same nest were clustered together but were not close to the extract of the paper of their nest (Figure 2). Eliminating pedicels from consideration and repeating the factor analysis yielded two factors that explain 52.3% of the variance, while all others contribute about 10% or less. In this case factor I is still mostly a contrast between shorter straight-chain alkenes and longer branched alkanes, but factor II appears to be primarily a

TABLE 3. FACTOR LOADINGS FOR AXES OF FIGURE 2

Peak	Component[a]	Factor loadings	
		Factor I	Factor II
1	*n*-C_{23}	−0.40	−0.17
2	*n*-C_{25}	−0.60	−0.39
3	*n*-C_{26}	−0.79	−0.42
4	*n*-C_{27}	−0.91	−0.22
5	Methyl-C_{27}	−0.93	0.16
6	3-Methyl-C_{27}	0.01	−0.51
7	*n*-C_{28}	−0.50	0.61
8	*n*-C_{29}	0.04	0.04
9	Methyl-C_{29}	−0.55	0.51
10	*n*-C_{30}	0.66	−0.44
11	*n*-C_{31}	0.54	−0.26
12	Methyl-C_{31}	−0.34	0.84
13	Dimethyl-C_{31}	0.87	−0.24
14	*n*-C_{32}	0.88	−0.16
15	Methyl-C_{33}	0.84	0.20
16	Dimethyl-C_{33}	0.36	0.38
	Unidentified	0.87	0.20
17	Methyl-C_{35}	0.25	0.71
18	Dimethyl-C_{35}	0.69	0.43
% variance explained		41.6	15.7

[a] Full names of components are listed in Table 2.

contrast between various hydrocarbons of 28–31 carbons, roughly the middle of the total range. The similarity of some wasp extracts to the extract of their nest paper was sometimes most obvious when the data were plotted on only one axis, either factor I or factor II (Figure 3).

DISCUSSION

Hydrocarbons were the only components detectable in the hexane extracts of *P. metricus* nests. These compounds would seem to be responsible for the increased rate of selection of their own nest by the wasps after application of the hexane extract to the nests (Table 1). Hexane extraction of the surface paper of the nests of the social wasps *Parachartergus aztecus* and *Polistes annularis* has shown that the nests have a surface layer of hydrocarbons that is also very similar in composition to that recovered from the cuticle of the respective wasps (Espelie and Hermann, 1988, 1990).

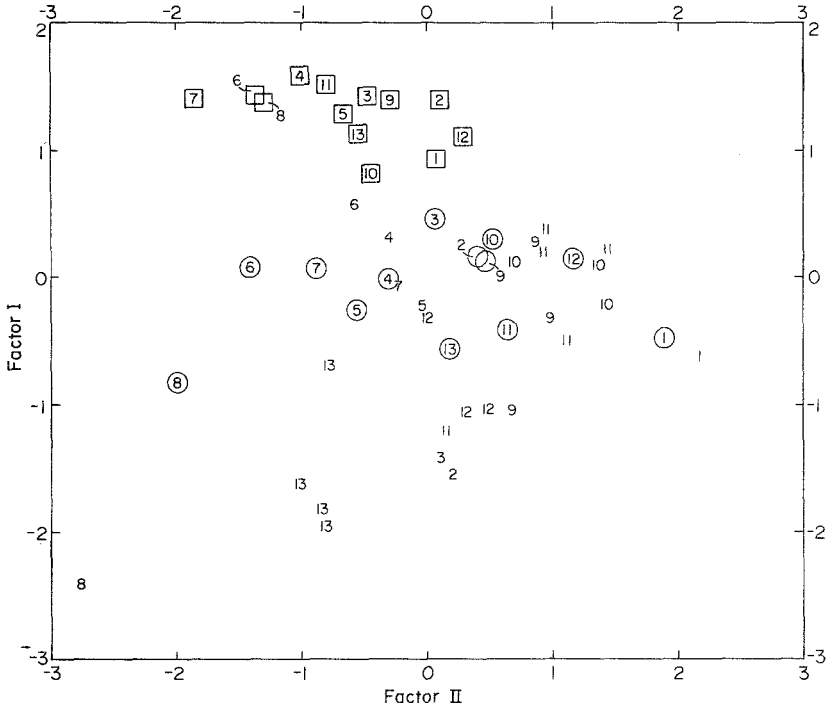


FIG. 2. Plot of factor I versus factor II for the extracts of *Polistes metricus* adults (isolated numbers), nest paper (circles), and nest pedicels (squares) from 13 nests.

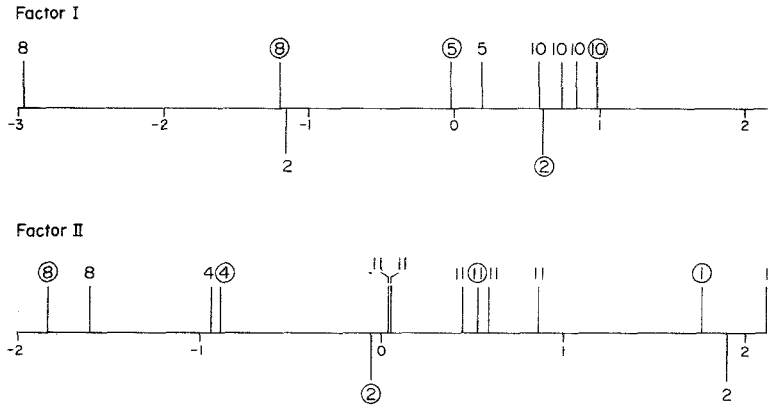


FIG. 3. Factor I and factor II values for the extracts of selected *Polistes metricus* adults (isolated numbers) and nest paper (circles). Note that factor scores are correlated for extracts from certain colonies, while other colonies, such as number 2 (below axes), have widely varying scores. These axes differ from those of Figure 2 in that pedicel extracts were excluded from the analysis.

The major hydrocarbons of *Polistes metricus* in Georgia are clusters of components with a backbone of either 27, 29, 31, or 33 carbons. This was also found for *P. annularis* adults (Espelie and Hermann, 1990). The dominant components recovered from the cuticle of *P. metricus* are C_{27} and C_{29} *n*-alkanes and C_{31} and C_{33} methylalkanes (Table 2), while the major hydrocarbons found in *P. annularis* adults are C_{27} and C_{29} 3-methylalkanes and C_{31} and C_{33} dimethylalkanes.

Variation in surface hydrocarbons may play an important role in nestmate recognition for *P. metricus*. The similarity in hydrocarbon profiles of some wasps to extracts of their nest paper (Figure 2) supports this possibility. The diversity of hydrocarbon profiles for the nest paper extracts and also for the wasp extracts (Figure 2) may be sufficient to allow nestmate discrimination. Although several wasps possessed hydrocarbon patterns distinct from those of their nest paper, it may not be necessary for a wasp to have surface chemistry identical to that of the paper. It is important to note in this regard that the colony that failed to favor its own nest after restoration of the hydrocarbons (Table 1) was tested against the carton of a colony that did favor its own nest in the reciprocal test. Thus it is not a case in which both colonies were confused, but rather that one did not discriminate and the other did.

It is possible that the wasps may utilize only some of the hydrocarbons in the recognition process. The cuticular lipids known from social wasps are a mixture of types of hydrocarbons, rather than merely a mixture of homologs with varying chain lengths (Table 2) (Espelie and Hermann, 1988, 1990). In the same way that some wasp and nest paper extracts show a closer association when plotting only one or another of the main factors (Figure 3), some wasps might recognize their kin primarily by the presence of a few traits rather than the mixture of all. By analogy, humans choose to recognize only the distinctive features in a face, the Roman nose or Hapsburg chin, not all uninformative features, such as the Roman chin or Hapsburg nose. Recently, Carlin (1989) has stressed the role of fortuitous idiosyncrasies in kin recognition.

The fact that there is little variation in the hydrocarbon composition of the *P. metricus* pedicels (Figure 2) supports the idea that this mixture serves a specific function. The pedicel must be strong enough to support the growing nest and must be waterproof. Jeanne (1970) originally reported that *Mischocyttarus drewseni* smears the pedicel of its nest with a secretion from the sternal gland, which acts as a deterrent to predators. This behavior has subsequently been observed for several genera of social wasps (Hermann and Dirks, 1974; Jeanne, 1975; Turillazzi and Ugolini, 1979; Kojima, 1982, 1983; Post and Jeanne, 1981). Methyl hexadecanoate has been found in the sternal gland of *P. fuscatus* workers and shown to act as a deterrent to ant predators (Post et al., 1984; Henderson and Jeanne, 1989). Hexadecanoic and octadecenoic acids have been recovered from the pedicel of *P. annularis* nests and may serve a similar

deterrent role (Espelie and Hermann, 1990). Furthermore, Wenzel (1989, 1990) has shown that traits associated with the pedicel or early construction tend to be stereotypical and less variable than other parts of the nest. Thus, while a great deal remains for further research, it appears that the compounds found on the surface of the pedicel will not prove to be those most important for problems of kin recognition.

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EXOCRINE SECRETIONS OF THE ANDROMEDA LACE BUG *Stephanitis takeyai* (HEMIPTERA: TINGIDAE)

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Abstract—The four major components of the setal exudate of nymphs of the andromeda lace bug, *Stephanitis takeyai*, were identified. They are 7-hydroxy-3-nonylchromanone **1**, the novel 3,5-dihydroxy-2-dodecanoylcyclohex-2-en-1-one **2**,2′,6′-dihydroxydodecanophenone **3**, and its 3-hydroxy derivative **4**. Only three species of *Stephanitis* commonly occur in the United States; with *S. takeyai* we have now identified the major compounds secreted by each of these species. The individual species of the genus are chemotaxonomically distinct and are characterized by the oxidation states of the secreted compounds.

Key Words—Hemiptera, Tingidae, *Stephanitis*, lace bug, setal exudate, acetogenins, oxidation state.

INTRODUCTION

The andromeda lace bug, *Stephanitis takeyai* Drake and Maa, is one of only a very few known insect pests of Japanese andromeda, *Pieris japonica* (Thunberg) (Dunbar, 1974). Nymphs of this insect, like those of other *Stephanitis* spp. and also *Corythucha* spp., exhibit, under magnification, numerous microdroplets of a clear fluid suspended at the ends of secretory setae, which are distributed over the body and antennae (Figure 1) (Livingston, 1978). For several years we have been investigating these secretions from both genera (Oliver et al., 1985, 1987; Lusby et al., 1987, 1989). From all species investigated, we have identified acetogenins consisting of an oxygenated six-member ring with a long (frequently 12 carbons) side chain, the latter also being oxygenated

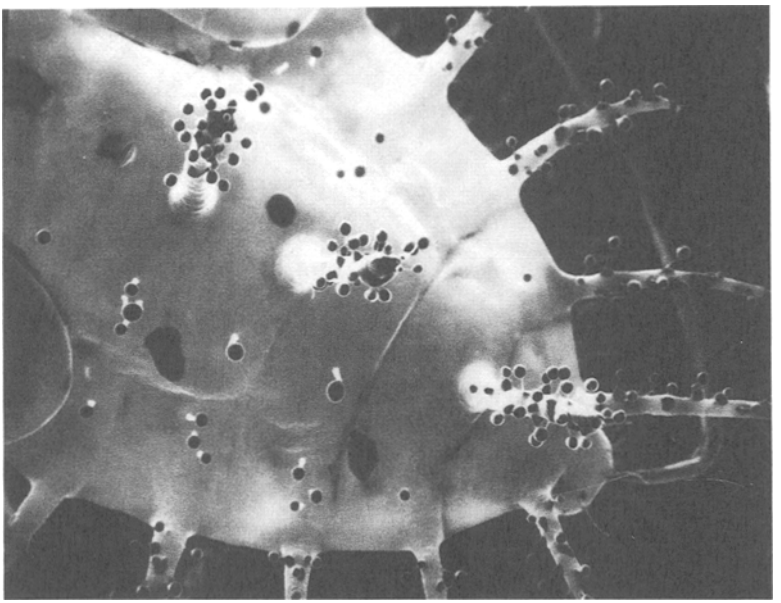


FIG. 1. Andromeda lace bug, *Stephanitis takeyai*, fifth-instar nymph. Dorsal abdominal view showing microdroplets containing secreted acetogenins (100×).

at the 1′ position and in some cases (*Stephanitis* only) at the 3′ position (Figure 2). To date, compounds identified from *Stephanitis* have had an aromatic ring with no multiple bonds or branches in the side chain (Oliver et al., 1985, 1987). In contrast, no aromatics have been encountered from *Corythucha*, and nearly all the compounds have been representative of the unusual 2-acyl-3, 6-dihydroxycyclohex-2-ene-1-one system with zero, one, or two double bonds in the (also unbranched) side chain (Lusby et al., 1987, 1989).

Henry and Froeschner (1988) report four species of *Stephanitis* observed in the United States. We had access only to three since *S. blatchleyi* Drake is found only in Florida and was last collected in 1927. We have previously reported our studies of *S. pyrioides* (Oliver et al., 1985) and *S. rhododendri* (Oliver et al., 1987); we here report our findings from the third species, the andromeda lace bug *S. takeyai* and compare the components of their secretions to those of the other species.

METHODS AND MATERIALS

Chromatographic procedures, mass spectrometry, and collection of lace bug exudates have been described previously (Oliver et al., 1985, 1987). HPLC collections were made from a 250 × 4.1-mm Hamilton PRP-1 "resin" column

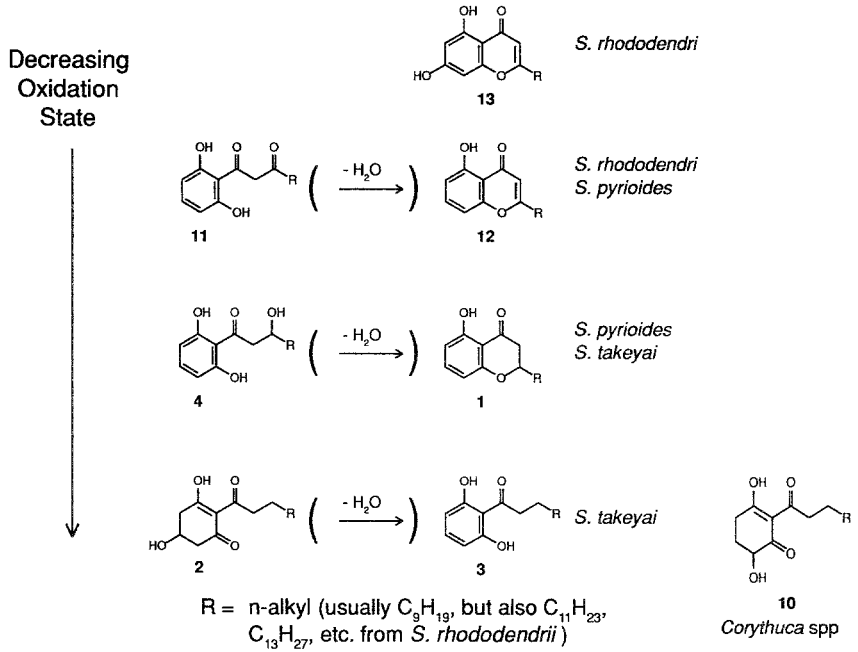


FIG. 2. Structural classes of lace bug acetogenins arranged in decreasing order of oxidation states.

eluted with 90% CH_3CN and 10% H_2O containing 0.1% HOAc, monitored at 220 nm. Exudates from individual lace bugs were obtained by rearing nymphs in isolation on single leaves of the host plant Japanese andromeda, *Pieris japonica* (Thunb.). The exuviae from the final two instars were placed in the bottom of a small conical vial and covered with CH_2Cl_2; the solvent was allowed to evaporate to a volume of a few microliters, and half or a third of the sample was used per (split) GLC injection. To assess influence of host plant on exudate composition, a group of nymphs were reared on leaves of an alternate host, azalea (Krume hybrid, "Blaauw's Pink").

Confirmation of Structure **2.** 2-Dodecanoyl-3-hydroxy-5-(phenyldimethylsilyl)cyclohex-2-en-1-one **5** was prepared according to Mudd (1985) from dodecanoyl chloride and 5-(phenyldimethylsilyl)cyclohexane-1,3-dione. The latter was synthesized from methyl (*E*)-3-(phenyldimethylsilyl)acrylate (Takeshita et al., 1987) and methyl acetoacetate under conditions similar to those of Crossley and Renouf (1915). A solution of **5** (34 mg) in HOAc (0.5 ml) was treated with 41 mg $Hg(OAc)_2$ and 0.5 ml of a solution of peracetic acid obtained by combining, at 0°C, 0.52 ml Ac_2O, 120 μl 30% H_2O_2, and 10 μl conc. H_2SO_4 (vigorous reaction). Samples were periodically withdrawn for GLC analysis (aliquots added to H_2O and products isolated via a C18 extraction cartridge).

Within 40 min at room temperature, ca. 28% of **5** had been converted to **2** (observed as a mixture of **2** and **3** because of dehydration during GLC analysis). No further conversion occurred, and addition of additional reagents resulted in the destruction of both **5** and **2** with dodecanoic acid as the only identifiable product. None of several variations on the procedure improved the conversion, and many seemed to inhibit it completely, e.g. the use of aqueous peracetic acid (Fleming and Sanderson, 1987). The material produced in this way was identical (GLC retention time and mass spectrum) to the major component of the *S. takeyai* secretion. Mass spectrum of **2**. *m/z* (%): 310, M^+(14), 292(6), 184(25), 183(100), 171(11), 170(63), 165(34), 155(28), 137(24), 134(10), 129(24), 128(11), 126(19), 111(17), 98(12), 97(15), 85(13), 84(11), 71(15), 69(58), 57(20), 55(47).

1-(2,6-Dihydroxyphenyl)dodecan-1-one **3.** A solution of 2-dodecanoyl-3-hydroxycyclohex-2-en-1-one **6** (Oliver and Lusby, 1988) (5.82 g), $Hg(OAc)_2$ (16 g) and NaOAc (3.8 g) in acetic acid (125 ml) was refluxed under N_2 6.5 hr. After concentration in vacuo, the residue was partitioned between H_2O and 4:1 hexane–EtOAc. The organic phase was washed with aq. $NaHSO_3$, dried (Na_2SO_4), and concentrated to give 6.16 g of crude product that was recrystallized from 80% MeOH, then from hexane, to give 3.8 g of **3,** mp 86–88°C. Mass spectrum: *m/z* (%): 292, M^+(3), 274(3), 189(9), 165(21), 152(21), 138(18), 137(100), 123(5), 81(7), 69(6), 55(11). UV (EtOH) 201(10100), 222(10900), 268(10400), 341(2700). [^{1}H]NMR ($CDCl_3$), 0.88(3H, t, J = 6.9, CH_3), 1.26(methylene envelope), 1.67(2H, m, H-3), 3.12(2H, t, J = 7.8, H-2), 6.39(2H, d, J = 8.4, H-3′ and 5′), 7.22(1H, t, J = 8.1, H-4′), 9.51(2H, s, OH).

1-(2,6-Dihydroxyphenyl)-3-hydroxydodecan-1-one **4.** Lithium diisopropylamide (LDA, 20 mmol) was prepared at −10°C under N_2 from diisopropylamine (3 ml) and *n*-butyllithium (2.5 M in hexane, 8 ml) in dry THF (10 ml). A solution of 10 mmol of the monobenzylether of 2,6-dihydroxyacetophenone **7** (Kametani and Kano, 1963) in THF (16 ml) and HMPA (4 ml) was cooled to −70°C under N_2, and the LDA solution was added dropwise by syringe. The pale yellow solution was stirred 1 hr while the temperature slowly rose to −55°C; it was then cooled to −75°C and treated dropwise with a solution of *n*-decanal (10 mmol) in THF (2 ml). The resulting mixture was stirred 40 min while the temperature rose to −60°C, then was quenched with a mixture of ice and aqueous NH_4Cl containing a little acetic acid. After extraction into ether, rinsing with water and aqueous $NaHCO_3$, drying, and concentration, 5.2 g of a clear oil was obtained that crystallized on standing. Recrystallation from heptane gave 2.71 g (68%) of 1-(2-benzyloxy-6-hydroxyphenyl)-3-hydroxydodecan-1-one **9** in two crops, mp 69.5–70.5. Mass spectrum (NH_3-CI): *m/z* 416($M + NH_4^+$). Mass spectrum, EI (*m/z*, %): 398(M^+, 1.9%), 290(6.1%), 200(3.5%), 163(24%), 137(26%), 91(100%), 65(8.6%), 55(6.9%).

A solution of **9** (461 mg) in 95% EtOH containing 50 mg 10% Pd on C

was hydrogenated for 15 min at 1 atm. Filtration and concentration gave 369 mg of a pale oil that was crystallized from heptane plus a few drops of EtOAc to provide pure **4** (305 mg, 85%), mp 81–82°C. EI-MS (*m/z*, %), 308(M^+, 10), 290(13), 163(100), 152(33), 137(97), 123(7), 108(12), 95(6), 81(20), 69(10), 57(16), 55(26). UV(EtOH) 202(10400), 221(10900), 270(10400), 343(2700). [^{1}H]NMR ($CDCl_3$) 0.88(3H, t, J = 7, CH_3), 1.27(methylene envelope), 3.06(1H, dd, J = 15.6 and 3.0, H-2), 3.38(1H, dd, J = 15.9 and 9.6, H-2), 4.33(1H, m, H-3), 6.43(2H, d, J = 8.4, H-3′ and H-5′), 7.26(1H, t, J = 8.3, H-4′).

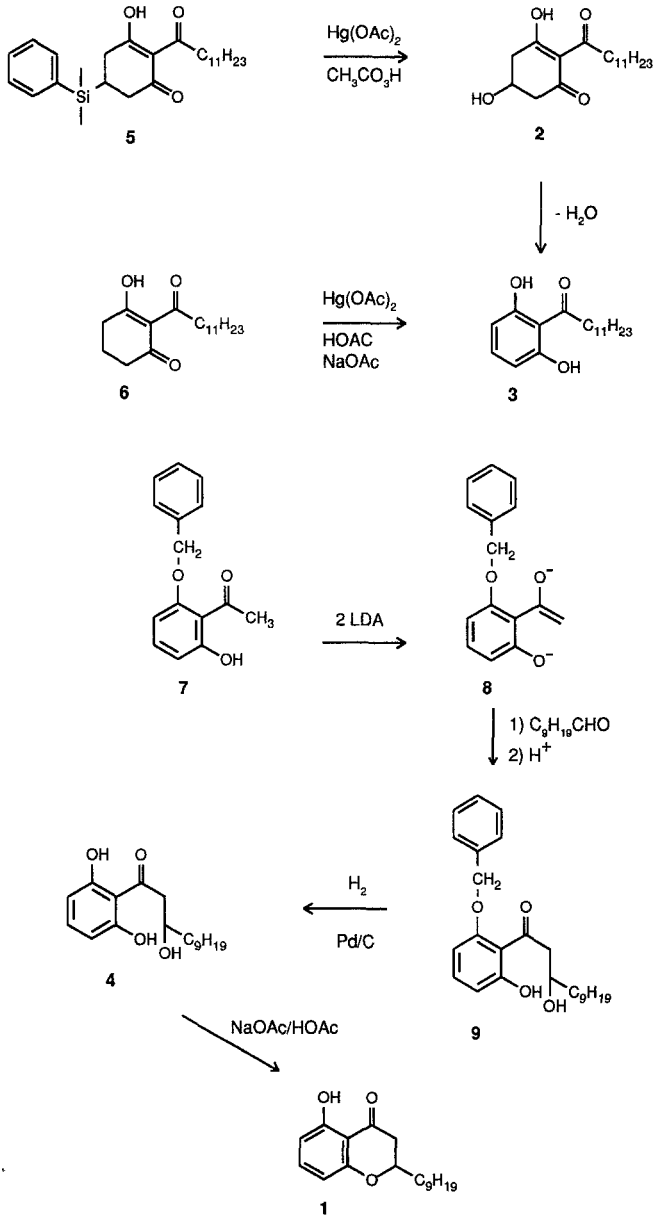
5-Hydroxy-2-nonylchromanone **1** from **4.** A solution of **4** (308 mg) and anhydrous NaOAc (250 mg) in acetic acid (10 ml) was heated at reflux 17.5 hr, then most of the acetic acid was stripped in vacuo and the residue was partitioned between H_2O and hexane–ether (1:1). The combined organic extracts were rinsed twice with H_2O, then with saturated $NaHCO_3$, and finally dried ($MgSO_4$) and concentrated. Flash chromatography on silica gel (5% EtOAc in hexane) of the residue provided 214 mg of pure **1** as a pale oil. [^{1}H]NMR($CDCl_3$) 0.88 (3H, t, J = 6.9, CH_3), 1.28(methylene envelope), 1.70(1H, m, H-1′), 1.86(1H, m, H-1′), 2.70(2H, m, H-3), 4.40(1H, m, H-2), 6.4–6.5(2H, m, H-6 and H-8), 7.31(1H, m, H-7).

RESULTS AND DISCUSSION

Gas chromatography (GC) and gas chromatography–mass spectrometry (GC-MS) of *S. takeyai* nymph setal exudates revealed three major components whose molecular weights and presumed molecular formulae were 290($C_{18}H_{26}O_3$) (**1**), 292($C_{18}H_{28}O_3$) (**2**), and 310($C_{18}H_{30}O_4$) (**3**), respectively. The first of these was 5-hydroxy-2-nonylchromanone **1**, which had previously been identified from the azalea lace bug, *S. pyrioides* (Oliver et al., 1985). RP-HPLC examination of the same mixture revealed four components, however. Preparative HPLC followed by GLC evaluation of the collected fractions revealed that **1** and **3** each gave single GLC peaks; however, **2** gave GLC peaks for both **2** and **3** (the ratios varying between GLC columns), indicating that partial dehydration of **2** to **3** was occurring at elevated temperature. Compound **4**, introduced into the mass spectrometer via probe instead of GLC, was determined to have a mol wt of 308; upon gas chromatography, however, it eluted exclusively as **1** (mol wt 290). In this case complete dehydration occurred.

Compound **3** was tentatively identified as a 2,6-dihydroxyacetophenone homolog by the close similarity of its mass spectrum to that of the 2,4-dihydroxy isomer, which had previously been synthesized in this laboratory (J. P. Kochansky, unpublished results). This assignment has been confirmed by synthesis (Scheme 1).

Compound **2,** which dehydrates to **3,** is isometric to, and produced a mass



SCHEME 1. Synthesis of new compounds from *S. takeyai*.

spectrum similar to, that of 2-dodecanoyl-3,6-dihydroxycyclohex-2-ene-1-one (**10,** R = n-C_9H_{19}). The latter compound was recently identified (Lusby *et al.*, 1989) from the secretion of the hawthron lace bug, *Corythucha cydoniae*, and synthesized (Oliver and Lusby, 1988). The apparent close similarity of **2** to **10a,** its cooccurrence with, and dehydration to, **3,** and the oxygenation patterns observed with *Stephanitis* compounds (Figure 2) suggested that **2** should be a 2-acyl-3,5-dihydroxycyclohex-2-ene-1-one. This would be the first nonaromatic acetogenin observed from this genus, and the first example of this class of compounds reported from any source.

Immediate support for proposed structure **2** was not readily available. Insufficient material was available for isolation and NMR analysis, and literature precedent was lacking for 3,5-dihydroxycyclohex-2-ene-1-ones (or their keto tautomers, 5-hydroxycyclohexane-1,3-diones), with or without a 2-acyl substituent. The 6-hydroxy isomer **10** (R = C_9H_{19}) is relatively stable and does not readily dehydrate (Oliver and Lusby, 1988), but **2** is a β-hydroxyketone and should be susceptible to facile elimination of water, especially in this case, where aromatization would occur. Consistent with this expectation, both **2** and **3** were detected by GLC in all samples investigated, but their ratios varied significantly when different gas chromatographs and/or columns were employed, a phenomenon consistent with dehydration of **2** to **3** occurring at elevated temperatures.

That the major component of the *S. takeyai* exudate is indeed the illustrated dihydroxycyclohexenone **2** was confirmed by generating it by the oxidative desilylation of the 5-phenyldimethylsilyl compound **5** by a modification of the Fleming and Sanderson (1987) procedure (Scheme 1). Thus, treatment of **5** with $Hg(OAc)_2$ and CH_3CO_3H plus a trace of H_2SO_4 gave, according to GLC and GLC-MS, a mixture of **2** and **3,** retention times and mass spectra of each being identical to those of the materials from the lace bugs. A competing reaction (generating lauric acid by side-chain cleavage) prevented this from being a satisfactory synthetic reaction; however, an alternative synthesis is being developed and will be reported separately.

The last of the four compounds eluted earliest from the reverse-phase HPLC column and dehydrated to **1** during GLC analysis. The simplest structure in keeping with the oxygenation patterns established thus far in the *Stephanitis* acetogenins (Oliver et al., 1985, 1987) would be the β-hydroxyacetophenone **4** (Figure 2). This structure also was confirmed by synthesis (Scheme 1). The monobenzyl ether of 2,6-dihydroxyacetophenene **7** (Kametani and Kato, 1963) was converted with two equivalents of lithium diisopropylamide to dianion **8,** which condensed with decanal at −75°C to give **9.** Catalytic hydrogenation converted **9** to **4** whose HPLC retention volume and mass spectrum were identical to those of the natural product. As expected, the retention time of synthetic **4** on GLC was indistinguishable from that of the dehydration product **1.**

Presumably **2** is in rapid equilibrium with its isomeric enol, in which case the asymmetry of C-5 is erased. Both **1** and **4** do have legitimate asymmetric centers, but in the absence of a bioassay their importance is difficult to assess and the question of absolute configuration has not be addressed.

In spite of the facile cyclodehydration of **4** during GLC analysis, it was reasonably stable to acidic reagents. For example, heating **4** in benzene containing a trace of trifluoroacetic acid did not affect the **4** → **1** transformation within 2 hr; the **4** → **1** reaction could be catalyzed by pyridinium *p*-toluenesulfonate, but was incomplete after 16 hr in refluxing toluene. On the other hand, refluxing **4** in acetic acid containing sodium acetate cleanly converted **4** to **1**; although this reaction also required a number of hours for completion, it constitutes a more convenient synthesis of **1** than our earlier version (Oliver et al., 1985) wherein competitive reduction of the carbonyl occurred during hydrogenation of the corresponding chromone **12.**

We reported that the exudate produced by the azalea lace bug, *S. pyrioides*, was independent of whether the insect was feeding on azalea or rhododendron leaves (Oliver et al., 1985). Similarly, *S. takeyai* exudates are indistinguishable whether the insect is fed on andromeda or azalea leaves.

All of the conclusions reported on *S. pyrioides* and *S. rhododendri* were reached from the study of pooled samples. More recently we have been able to examine exudates of individual insects of both *S. takeyai* and *S. pyrioides* and have observed no significant variations between individuals of the same or opposite sex of either species. From GLC analysis of pooled cast skins of fifth-instar *S. takeyai*, we estimated total amounts of **1–4** to be ca. 1.1 μg/nymph (the nymphs themselves probably weigh $\leq$400 μg).

An overview of the structural types identified from the genus *Stephanitis* is presented in Figure 2. From a biosynthetic point of view, the number of significant components may be fewer than illustrated since **1, 3,** and **12** are simply dehydration products of **4, 2,** and **11,** respectively, and may result from nonenzymatic processes.

The similarity of the compounds and the biosynthetic implications are noteworthy, but equally interesting is the distribution according to oxidation state. Even though certain structures (**1, 11, 12**) are shared by two species, and, in fact, the azalea lace bug, *S. pyrioides*, has no major components exclusively its own, each species is, in a sense, defined by the two adjacent oxidation states its secretions occupy. Thus, of the acetogenins secreted by the three species in question, those of *S. rhododendri* occupy the highest two oxidation states, whereas those of *S. pyrioides* are found in the second and third and those of *S. takeyai* in the third and fourth highest states. The secretion of *S. rhododendri* further differed in containing a larger number of compounds (Oliver et al., 1987), but these were simply homologs of the same general structures with side chains of different lengths.

Oxidation state differences are seen within the *Corythucha* secretions too, but in these cases by virtue of zero, one, and two double bonds in the side chain of general structure **10.** Our investigations to date, admittedly not exhaustive because of the large number of *Corythucha* species, indicate that the oxidation state distribution is less systematic, with any species likely to have representatives of **10** with zero, one, or two double bonds.

Our investigations of the functions of the secreted products have to date been less conclusive than those of their structures. The abundance of the microdroplets, the gregarious nature of the nymphs, and their apparent lack of natural enemies (Sheeley and Yonke, 1977) are strongly suggestive of a protective function, but this remains to be demonstrated unequivocally, the synthetic materials seeming to lack any obvious toxic or repellent properties toward other insects. Curiously, the alkanophenone **3** very actively inhibited two prostaglandin synthase enzymes in in vitro assays (Jurenka et al., 1989), but too little work has been done to fully assess the generality of significance of this phenomenon.

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EFFECT OF DIACETYL PIQUEROL ON H^+-ATPase ACTIVITY OF MICROSOMES FROM *Ipomoea purpurea*[1]

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Abstract—The effect of an allelopathic compound, diacetyl-piquerol on the H^+-ATPase activity of the microsomal fraction from the radicles of a common weed *Ipomoea purpurea* was studied. The diacetyl-piquerol inhibited the germination and radicle growth from *I. purpurea*; the radicle growth was increasingly inhibited (10% to 100%) as piquerol concentrations were raised (10 μM to 1000 μM). The H^+-ATPase activity was inhibited (48%) by 500 μM diacetyl-piquerol, and this inhibition was higher in plasma membrane ATPase (67.2%) than in tonoplast membrane ATPase (31.4%). Additional studies of the precise physiological mechanisms of interference caused by allelopathic compounds are needed.

Key Words—Piquerol, *Piqueria trinervia*, modes of action, allelopathy, allelochemicals, microsomes, *Ipomoea purpurea*, plasmalemma, tonoplast, germination, H^+-ATPase, proton pumps.

INTRODUCTION

One of the main objectives in a study of allelopathy is to know the ecological role of the allelochemicals produced and released by donor plants into the environment. These compounds have an influence on the plants in a community,

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through effects on growth, development, and vigor, as a result of interference with metabolic processes (Horsley, 1977).

Knowledge of the modes of action of allelopathic compounds is scarce because many compounds are known, there is a wide array of cellular processes with which they may interfere, and few investigators have followed allelopathic phenomena to the biochemical level. Clear insight into the precise physiological alterations caused by these substances has not been obtained (Horsley, 1977; Einhellig, 1986). Studies on mechanisms of allelochemical inhibition have shown that allelochemicals can act at several sites within a plant cell, altering rate of ion uptake, synthesis of proteins, activation or breakdown of hormones, photosynthesis, energy metabolism, enzymatic activity, respiration, and cell division (Cameron and Julian, 1980; Rice, 1984; Uribe et al., 1985; Balke, 1985; Moreland and Novitsky, 1986; Cruz et al., 1988).

Piqueria is one of the genera of the Eupatorieae that has produced many important secondary compounds, some of them having practical uses (Domínguez, 1977; Romo et al., 1970). *Piqueria trinervia* grows in the central part of Mexico, forming pure populations of different sizes. Romo et al. (1970) isolated from this species two monoterpene diasteroisomers, piquerol A and piquerol B, which were tested by González de la Parra et al. (1981) in vitro as possible allelophatic substances. In this study, extracts of roots and leaves, as well as purified piquerol A and B produced a strong inhibitory effect on the radicle and stem growth of six test weed species. On the other hand, Cruz-Reyes et al. (1989) showed the molluscicide activity of piquerol A on eight species of pulmonar snails.

In some cases, allelopathic effects are more obvious on roots than on stems of test seedlings (Anaya and Rovalo, 1976; Anaya, 1976; Anaya and del Amo, 1978; Anaya et al., 1982). Many vascular and cellular transport processes take place in the root, where many of the solute fluxes through the cellular membranes are mediated by protein translocators. Primary pumps, or H^+-ATPases are transmembrane proteins that provide the formation of electrochemical proton gradients at the expense of ATP hydrolysis. These gradients drive the transport of ions, amino acids, sugars, and other molecules through plant cell membranes (Marré and Ballarin-Denti, 1985; Serrano, 1988). Transport and enzymatic systems located in cell membranes may be one possible site of action for allelopathic natural products. The purpose of this work was to determine whether membrane H^+-ATPases were inhibited by the monoterpene compound piquerol. We tested the effect of this compound in microsomal fractions of the common weed *Ipomoea purpurea* L., morningglory (Convolvulaceae). The microsomal vesicles contained the tonoplast and plasmalemma and their respective H^+-ATPases.

METHODS AND MATERIALS

Piquerol A was obtained from dry leaves of *Piqueria trinervia* and subjected to further acetylation (100 mg of piquerol A were dissolved in 1 ml of anhydrous pyridine, 1 ml of recently distilled acetic anhydride added, the solution purified by distillation, and the acetate of piquerol was obtained as a colorless oil) (Figure 1). This chemical modification was done in order to trace the molecule for further chemical studies. The acetylated compound showed essentially the same allelopathic action as piquerol A (Table 1).

Bioassay with Piquerol A and Diacetyl-piquerol. Ten seeds of *I. purpurea* were placed in Petri dishes with filter paper (a semisterile system) supplemented with different piquerol-A and diacetyl-piquerol concentrations in a range of 10–100 ppm. Another bioassay with only diacetyl-piquerol using concentrations in a range of 10 to 1000 μM, was made. The compounds were dissolved in ethanol, added to 5 ml of distilled water, and poured on a Petri dish with filter paper (final concentration of ethanol never exceeded 0.1%). Appropiate controls were done with water and ethanol. Seeds were germinated at 24°C in darkness, and after 48 hr radicle length was measured. Each treatment was repeated four times. Data were statistically analyzed (ANOVA) as a randomized complete block design.

Microsomal Fraction. I. purpurea seeds were germinated in damp paper towels at 24°C in dark. After four days of growth, roots were excised, cut, and homogenized with a polytron for 15 sec in a buffer containing 250 mM Sucrose, 70 mM Tris HCl, pH 8.0, 2 mM EDTA, 1% BSA, 1 mM Na_2ATP, 15 mM 2-mercaptoethanol, 4 mM ditiotreithol, 2 mM PMSF (phenyl methyl sulfonyl fluoride), and 3 mM of $MgCl_2$. The filtered homogenate was centrifuged at 500g for 10 min at 4°C, and the supernatant at 13,000g for 10 min at 4°C. This supernatant was spun down at 80,000g for 30 min. The microsomal pellet was resuspended in 250 mM sucrose, 5 mM Tris HCl, pH 7.0, 0.2% BSA, 3.7% glycerol, 1 mM PMSF and 1 mM dithiotreithol (DTT), and stored at −70°C in several aliquots until use for ATPase activity determinations.

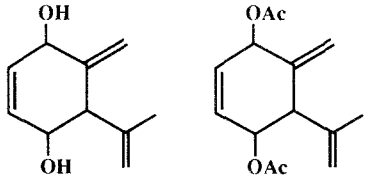


FIG. 1. Structures of piquerol A and diacetyl-piquerol.

TABLE 1. EFFECT OF PIQUEROL A AND DIACETYL-PIQUEROL ON RADICLE GROWTH OF *I. purpurea*[a]

Treatments (ppm)	Inhibition (%) of radicle growth
Piquerol A	
10	65.7
25	76.5
50	72.9
100	81.9
Diacetyl-piquerol	
10	46.4
25	63.3
50	100
100	100

[a] All numbers are significant at 1% level.

ATPase Activity Measurement. Microsomal protein (10 μg) was added to 150 μl of reaction medium consisting of 250 mM sucrose, 20 mM Tris HCl, pH 7.0, 10 mM ATP, 10 mM $MgCl_2$, and 7 μM CCCP (carbonyl cyanide *m*-chlorphenylhydrazone). When required, the following inhibitors were supplemented: 2 mM Na_2N_3 to inhibit mitochondrial ATPase (Gallager and Leonard, 1982), 2 mM $Na_2MoO_4 \cdot 2H_2$ to inhibit acid phosphatase (Admon et al., 1981), 200 μM Na_2VO_4 to inhibit plasma membrane ATPase (Colombo et al., 1981), and 100 mM KNO_3 to inhibit tonoplast ATPase (Ullah and Gibson, 1988). Reaction was allowed to proceed at 30°C during 20 min, and it was arrested by addition of 6% sodium dodecyl sulfate (SDS). Phosphate release was estimated by the method of Chifflet et al. (1988). Phosphate produced from ATP hydrolysis was from 114 to 227.8 nmol Pi/min/mg in different microsomal preparations.

Other Methods. Critical micellar concentration of diacetyl-piquerol was estimated using the fluorescent dye 1,6-diphenyl 1,3,5-hexatriene (DPH) as described by Chattopadhyay and London (1984). No micellar formation was detected by this method.

Protein determination used the method of Peterson (1977).

RESULTS

We initially established that the monoterpene diacetyl-piquerol had similar allelopathic effect as the nonacetylated compound on the *I. purpurea* growth (Table 1). Figure 2 shows the effects of 25, 50, and 500 μM of diacetyl-piquerol

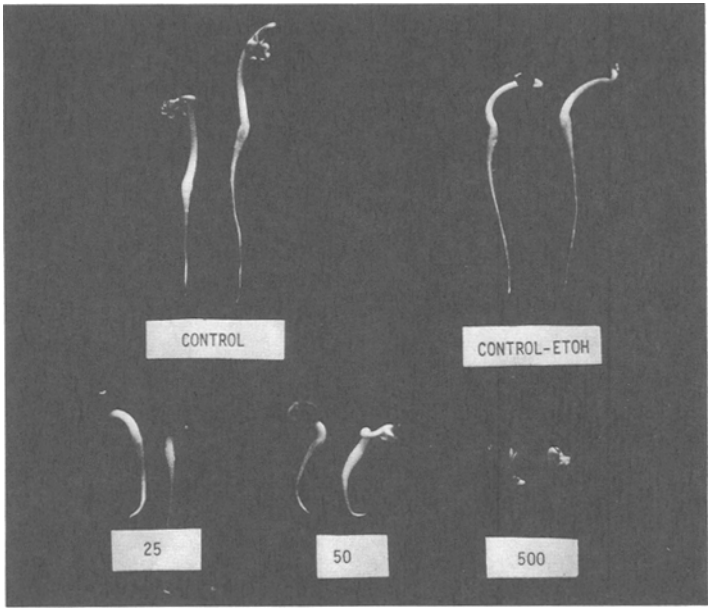


FIG. 2. Effect of three concentrations of diacetyl-piquerol on the growth of *Ipomoea purpurea* seedlings (500 μM correspond to 166.6 ppm).

on the growth of *I. purpurea* seedlings. Significant inhibition of germination (15%) was achieved at 50 μM diacetyl-piquerol; germination was totally suppressed at 1000 μM; radicle growth inhibition increased as the diacetyl-piquerol concentration increased (Figure 3).

These effects of diacetyl-piquerol on radicle growth were correlated with effects on microsomal fractions of *I. purpurea* radicles. Table 2 shows ATP hydrolysis in microsomal fractions incubated with 250–1000 μM of diacetyl-piquerol. Maximum inhibition (48.5%) was at 500 μM. This pattern was reproduced in five experiments.

Since ATP hydrolysis was measured under conditions where acid phosphatase and mitochondrial ATPase activities can be expressed (Table 2), we carried out ATPase activity quantitation in the presence of specific inhibitors to make more precise estimates of the effect of diacetyl-piquerol on ATPase activities of the plasma and tonoplast membranes (Table 3).

The results show that ATPase activity of the plasma membrane and tonoplast ATPase contribute 67.5 and 36.1% of the total activity, respectively (considering a control of 227.8 nmol Pi/min/mg, see head of Table 3). The effect of 500 μM diacetyl-piquerol was greater on plasma membrane ATPase activity, with 67.2 inhibition, than on the tonoplast ATPase activity (31.6%) inhibition.

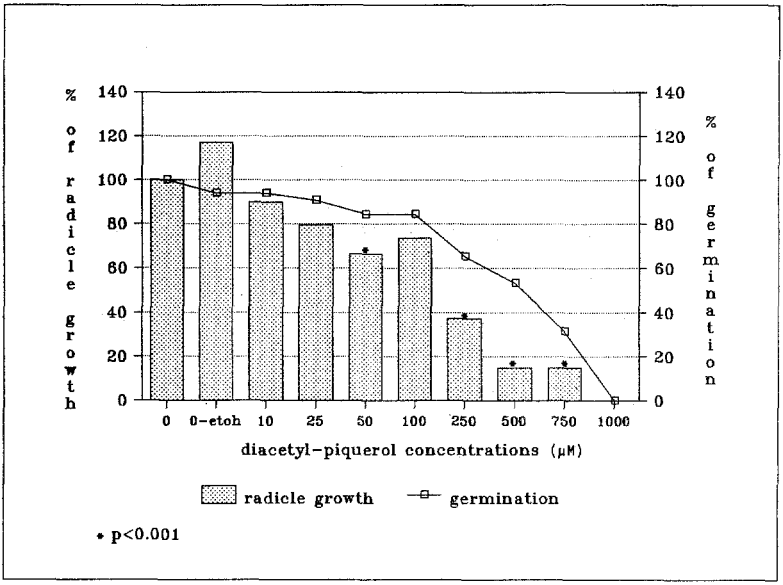


FIG. 3. Effect of diacetyl-piquerol on growth and germination of *Ipomoea purpurea* (significance refers to radicle growth).

TABLE 2. EFFECT OF DIFFERENT DIACETYL-PIQUEROL CONCENTRATIONS ON ATP HYDROLYSIS OF MICROSOMAL FRACTION FROM *I. purpurea* RADICLES

Diacetyl-piquerol (μM)	Hydrolysis (nmol Pi/min/mg)	Inhibition (%)
0	170.3	0
10	164.4	4
25	170.2	0
50	171.4	0
100	170.6	0
250	140.9	17.4
500	87.7	48.5
750	116.9	31.4
1000	111.5	34.5

DISCUSSION

Many allelopathic compounds are absorbed from the soil by the roots, the first plant organ to contact them. It is reasonable to hypothesize that the cell wall and the plasma membrane may be one of the initial targets of these com-

TABLE 3. EFFECT OF DIACETYL-PIQUEROL ON ACTIVITY OF MICROSOMAL ATPases FROM *I. purpurea* RADICLES[a]

ATPase activity origin	ATPase activity	
	nmol Pi/min/mg	Inhibition (%)
Plasma membrane ATPase	153.7	0
Plasma membrane ATPase + 500 μM diacetyl-piquerol	50.4	67.2
Tonoplast ATPase	82.2	0
Tonoplast ATPase + 500 μM diacetyl-piquerol	56.2	31.6

[a] Total microsomal ATPase activity was determined in the absence of any inhibitor and was 227.8 nmol Pi/min/mg. Plasma membrane ATPase activity was measured as Pi released in a media including NO_3, N_3, and Mo (see Methods and Materials) in the absence and presence of 500 μM diacetyl-piquerol. Tonoplast ATPase activity was determined in a similar way but in the presence of VO_4, N_3, and Mo (see Methods and Materials), and in the absence and presence of 500 μM diacetyl-piquerol.

pounds. In fact, Balke (1985), found that flavonoid compounds inhibit the ATPase activity of plasma membranes isolated from oat roots. In the present study we established that the allelopathic compound diacetyl-piquerol is able to inhibit seed germination and root growth of the weed *I. purpurea*. Several biochemical systems may be affected by diacetyl-piquerol, since the processes affected by the substance depend on the expression of many enzymatic activities. We focused our experiments on the activities of H^+-ATPase from different membranes, since it is an important enzyme involved in transport processes (Sze, 1985).

We found that the ATPase activity of the tonoplast and plasma membrane were inhibited by diacetyl-piquerol. It is interesting that the plasmalemma ATPase showed the major inhibition as compared to the H^+ATPase located in the tonoplast, because in vivo the plasma membrane ATPase might well be a target more accessible to the inhibitor than the tonoplast ATPase, given their respective cellular locations. These data suggest that the action of diacetyl-piquerol in vivo could be related to some extent to the effect on the plasma membrane H^+-ATPase.

The mechanism of diacetyl-piquerol inhibition is not known. We explored the possibility of membrane solubilization by diacetyl-piquerol micelles; however, we determined that this compound does not present measurable micellar formation (results not shown). Also, we have discarded the possibility of an uncoupling effect of diacetyl-piquerol because ATP hydrolysis has been measured under uncoupled conditions and because the compound does not prevent uncoupling in other biological membranes (Anaya, 1989). The effect could be

due to direct interaction of diacetyl-piquerol with H^+-ATPase or with the immediate lipid environment of the enzyme.

We found that significant inhibition of radicle growth, germination, and microsomal ATPase activity was attained at different diacetyl-piquerol concentrations, i.e., 50 μM and 500 μM, respectively. This suggests that several mechanisms of inhibition may be involved. Unfortunately, no information is available about the concentrations that allelopathic compounds reach in the soil and plant cell. Additional studies of the modes of action of allelopathic natural products, including diacetyl-piquerol, are needed.

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SOURCE AND NATURE OF DISTURBANCE-CHEMICAL SYSTEM IN CRAYFISH

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Abstract—The responses of crayfish to water from aquaria containing either undisturbed or disturbed animals were observed. The crayfish *Orconectes propinquus* and *O. rusticus* showed no response to disturbed-conspecific water. Individuals of *O. virilis* respond not only to disturbed crayfish but to other taxa (the leech *Macrobdella decora*, the darter *Etheostoma exile*, and rock bass *Ambloplites rupestris*), but not to the painted turtle *Chrsymes picta*. Additional tests indicated partial responses by *O. virilis* to ammonium and to a chemical or chemicals released from the green gland of crayfish. Ablation experiments indicated the antennules as the site of reception of the chemicals. Additional behavioral tests indicated that detection of the disturbance chemical(s) results in the crayfish showing low-level alert for more than 15 min, once an initial priming period has passed.

Key Words—Disturbance, semiochemical, communication, crayfish, *Orconectes propinquus*, *O. rusticus*, *O. virilis*, cross-taxa effects, pheromone, ammonium, antennules, green gland.

INTRODUCTION

Alteration of behavior upon detection of chemical signals given off by organisms belonging to different taxa is a widely recognized phenomenon in recent years. In many cases, the detection of a potential predator alters the behavior of potential prey (Petranka et al., 1987; Stemberger and Gilbert, 1987). In other cases, closely related animals may utilize each other's signals (Hazlett, 1985b), perhaps as a result of phylogenetic inertia. In the current study, the diversity of both sources and recipients of a chemical signal were investigated.

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The chemical communication system of interest is the disturbance "pheromone" of the crayfish *Orconectes virilis* (Hazlett, 1985a). When conspecific individuals are disturbed (made more active by any of several means) but not injured, individuals of *O. virilis* show a set of postures that are associated with low-level alert. The postures shown are intermediate between the resting postures of the crayfish and those postures associated with specific sources of danger or disturbance to the crayfish. In addition to conspecifics, individuals of other species (Hazlett, 1985b) and genera (Hazlett, 1989) of crayfish can serve as sources of the chemical. Attempts to demonstrate that animals in more distant taxa could serve as sources of the chemical were not successful (Hazlett, 1989).

In addition to looking at the diversity of sources and recipients of this chemical, the present study also looks at several aspects of the changes in behavior elicited by the perception of the pheromone. The sites of release and detection of the pheromone in crayfish were also studied.

METHODS AND MATERIALS

Experiments were conducted at the University of Michigan Biological Station, Pellston, Michigan, during July and August, 1988 and 1989. In general, methods were identical to those described earlier (Hazlett, 1985a,b, 1989). The crayfish tested (*Orconectes virilis*, *O. propinquus*, and *O. rusticus*) were housed individually in 10-gallon aquaria (26 × 51 cm bottom area) that were visually isolated and had a water depth of 10 cm. Each test crayfish was provided with a clay burrow, and the water in the aquarium was fully aerated at all times. An individual was tested with just one type of water per day. Water to be tested was introduced via a peristaltic pump at the rate of 20 ml/min for a test period of 8 min, except where noted.

The behavior patterns recorded have been described earlier (Hazlett, 1985a,b, 1989) and involved three positions (raised, neutral, lowered) of three body parts (chelipeds, cephalothorax, abdomen). All observations were done between 0800 and 1400 hr, a time of day when the crayfish are normally quiescent, resting in a fully lowered posture, usually at the entrance of their burrow. Earlier work showed that the primary response by crayfish to disturbance chemicals was the assumption of a neutral posture. In this posture the abdomen is partially extended with the uropods approximately perpendicular to the substratum, the walking legs extended partially such that the carapace is clearly not touching the substratum, and the chelipeds are partially extended with the tips of the dactyls lightly touching the substratum. The data compared in this study are the number of seconds spent by test crayfish with any body part in the neutral position when various sources of water were introduced into the aquaria via a clear plastic tube connected to a perstaltic pump. In some cases, the num-

ber of seconds spent in the fully lowered posture also was compared. In most cases, paired *t* tests were run to see if the responses of individual crayfish were different under different conditions. ANOVA was used to examine the results from the duration-of-effect test (see below) and unpaired *t* tests were used for the ablation experiments.

Other Animals as Sources. Earlier studies (Hazlett, 1985b, 1989) showed that individuals of *Orconectes virilis* responded to disturbance chemicals from a variety of types of crayfish (conspecifics, at least two congeners, and at least one *Cambarus* species) as well as suggesting a response to other types of aquatic animals. To further investigate the possibility that crayfish respond to a chemical or chemicals given off by disturbed (but not injured) animals in other taxa, four aquatic species sympatric with *O. virilis* were tested as source animals. Continually aerated water from aquaria containing undisturbed or disturbed (by simulated predator chase every 30 sec during the observation period, no physical damage inflicted) individuals was introduced to individual *O. virilis* and the postures of the crayfish recorded for 8 min on a PC event recorder. The source species tested were (1) 12 individuals of the darter, *Etheostoma exile*, (2) four large individuals of the leech, *Macrobdella decora*, (3) two individuals of the rock bass, *Ambloplites rupestris*, and (4) one individual of the painted turtle, *Chrsymes picta*. The rock bass was tested only in the "disturbed" situation as it was not possible to get those fish to be fully undisturbed in the laboratory.

Sources of Pheromone in Crayfish. The fact that the disturbance pheromone is given off by metabolically active crayfish suggested the possibility that the green gland could be the site of release of the pheromone. Alternatively, the fact (from above and earlier work) that all source species that elicit a response are ammonotilic and the species that did not elicit a response (painted turtle) is ureotilic suggests ammonia/ammonium ion as a possible basis of the reponse. Water samples (pH 8.3) analyzed on a Techicon Autoanalyzer II showed an increase in ammonium from about 30 μg/liter from aquaria with undisturbed crayfish to 250 μg/liter in aquaria with disturbed crayfish. (The behavioral tests mentioned above were done in 1988, prior to the suggestion that ammonium might be involved, thus no measurements of ammonium concentration were made.)

Based upon the above, a set of individual *O. virilis* were tested with the following sequence of sources: (1) self water—as a control and to establish a baseline of behavior (see Rose, 1986, for a discussion of this type of control), (2) standard *O. rusticus*—two individuals of *O. rusticus* in about 8 liters of water were repeatedly agitated during the 8 min delivery/observation period, (3) ammonium—a solution of 200 μg/liter ammonium chloride in filtered lake water, solutions made up daily and used within an hour after mixing, (4) plugged green gland—the orifices of the green glands of two individuals of *O. rusticus*

were covered over with cyanoacrylate glue; these disturbed crayfish were rinsed in running lake water and placed in 4 liters of water to serve as a source of disturbance pheromone, (5) control glue—two individuals of *O. rusticus* had cyanoacrylate glue spread over portions of their carapace, were rinsed off and placed in 4 liters of water to serve as a source of disturbance pheromone. It should be noted that the primary site of ammonia release by crayfish is the gills, rather than the green gland.

Reception of Pheromone. To examine the possible site of pheromone reception, ablations were performed on 20 individuals of *O. virilis*. Ten individuals had both antennae removed, and 10 individuals had both antennules removed. All operated crayfish were allowed to recover for 48 hr and then tested with water from disturbed individuals of *O. rusticus*.

Nature of Response by O. virilis. It was noted in earlier studies (Hazlett, 1985b) that there appeared to be a marked delay of about 2 min between the introduction of test water and any response by target crayfish. Two experiments were done to see if this delay was due to the need to exceed some threshold concentration of the pheromone in the surrounding water. In the first experiment, water from an aquarium with either (1) one disturbed *O. rusticus* or (2) five disturbed *O. rusticus* was introduced to individual *O. virilis*, and the time until a response (=change in behavioral postures) was shown was recorded. In the second experiment, water from an aquarium containing two disturbed *O. rusticus* was introduced to individual *O. virilis* at a rate of either (1) 20 ml/min or (2) 60 ml/min. The latency until a response was shown was recorded.

The second type of experiment concerning the nature of the response looked at how long the behavior of recipient crayfish was modified by detection of the chemical(s). Water from two disturbed *O. rusticus* was introduced at 20 ml/min for 6 min. At the end of this time, water delivery stopped and data on the postures of the recipient crayfish were recorded for 15 min. During control periods, self water was introduced and the crayfish's behavior followed for 15 min after the cessation of self-water introduction. In subsequent analyses, the data were divided into three 5-min periods and ANOVA used to test for changes in the difference of test and control situations over time.

The third type of experiment examined how perception of the disturbance chemical by individual *O. virilis* altered their responsiveness to other types of stimuli. Crayfish were exposed to either self water or water from an aquarium with disturbed *O. rusticus* for 5 min. The crayfish then were exposed to one of two types of stimuli and the latency of response measured. The light stimulus consisted of flashes from a Thyristor electronic flash placed next to the aquarium, facing the crayfish, and discharged once every 5 sec. The food stimulus was water containing a macerated piece of fish (rock bass) delivered at the rate of 25 ml/min. The food-related response to fish odor was qualitatively different from the responses shown to disturbance pheromone in terms of both the pos-

tures assumed (chelae raised, abdomen fully extended) and the greater speed of locomotion following the food odor. Ten crayfish were tested once with each type of stimulus under primed (from delivery of disturbance-chemical water) and control conditions.

Responses of Other Species of Crayfish. To examine the generality of the responses to disturbance pheromone shown by individuals of *Orconectes virilis*, individuals of two other species were tested. Water was introduced at the rate of 20 ml/min for 8 min. Specimens of *O. propinquus* were collected from the Maple River and the postures recorded as water from undisturbed and disturbed (by simulated predator chase) specimens of (1) *O. propinquus* and (2) *O. virilis* was introduced. Specimens of *O. rusticus* were collected from Burt Lake and tested with water from undisturbed and disturbed conspecific specimens.

RESULTS

The results of tests using sources of the pheromone other than crayfish are shown in Table 1. Considering the amounts of time spent in neutral and the full down positions by recipient crayfish, both the darters and the leeches clearly elicited a response on the part of *O. virilis* when they were disturbed. When the darters or leeches were disturbed, the crayfish spent more time in the neutral postures and less time in the full down posture than when water from undisturbed darters or leeches was introduced. While the response appeared to be less intense or reliable than that evoked by water from disturbed crayfish (Hazlett, 1985a), there were very significant differences in crayfish behavior depending upon the condition of the animals belonging to the other taxa. This was not true of the painted turtle. Although the data tended to vary in the same

TABLE 1. AVERAGE (±SD) NUMBER OF SECONDS DURING 8-MINUTE OBSERVATION SPENT BY *O. virilis* WITH ONE OR MORE PARTS IN NEUTRAL AND FULL DOWN POSTURE WHEN WATER FROM VARIOUS SOURCES (SAMPLE SIZES IN PARENTHESES) WAS INTRODUCED[a]

	Neutral postures			Full down posture		
Source water	Undisturbed	Disturbed	*P*	Undisturbed	Disturbed	*P*
Darters (19)	98 (±134)	170 (±116)	0.109	342 (±160)	186 (±131)	0.002
Leeches (20)	89 (±68)	192 (±122)	0.002	309 (±150)	187 (±136)	0.001
Turtle (14)	84 (±91)	136 (±112)	0.157	340 (±148)	235 (±154)	0.058
Bass (19)		285 (±137)			138 (±143)	

[a] *P* values associated with paired *t* test comparing responses to water from undisturbed and disturbed source animals.

general way as with the other taxa, there were no significant differences in postures with disturbed and undisturbed *C. picta*.

In an earlier study (Hazlett, 1989), evidence for differential responses by crayfish to disturbed and undisturbed noncrustaceans was equivocal. In the case of the red-spotted newt (*Notopthalamus viridescens*), crayfish responded to disturbed-animal source water as if the water contained a toxin. There was no significant difference in responses to water from disturbed and "undisturbed" catfish (*Ictalurus natalis*). The latter result, in contrast to the results reported for darters and leeches in the current study, was probably due to the lack of really undisturbed source animals. While the catfish used in the earlier study seemed to settle down in the laboratory rather well, they always displayed some level of "nervous" activity. In the case of both darters and leeches (and painted turtles), the undisturbed animals were very quiescent. As noted earlier (Hazlett, 1989), unless both source and recipient animals can be observed in an undisturbed state, detection of a response to disturbed-animal water is not possible.

The responses shown to the disturbed rock bass could not be compared directly to undisturbed rock bass because these fish were sufficiently jumpy in the laboratory that obtaining water from undisturbed individuals proved very difficult. The reponses of crayfish to water from rock bass were compared with two other sources of the chemicals, the crayfish *O. rusticus* and the darters. The rock bass evoked significantly more seconds spent in the neutral postures than the disturbed darters ($t = 2.88$, $P < 0.01$) and were not significantly different from the pattern evoked by the crayfish *O. rusticus* ($t = 1.2$, $P > 0.10$). The relatively high level of response to disturbed rock bass may be associated with the fact that the fish were disturbed for several days (while in the laboratory situation) rather than just the few minutes before a set of tests.

The results of the tests on the source of the pheromone in crayfish are shown in Table 2. While a number of comparisons are possible here, the essential information derives from two comparisons for each set of data. Use of the

TABLE 2. AVERAGE (±SD) NUMBER OF SECONDS DURING 8-MIN OSERVATION SPENT BY *O. virilis* WITH ONE OR MORE PARTS IN NEUTRAL AND FULL DOWN POSTURE WHEN WATER FROM VARIOUS SOURCES WAS INTRODUCED[a]

Source of water	Neutral postures	Full down posture
Self water	39 (±76)	440 (±77)
Standard	234 (±131)	232 (±145)
Ammonia	131 (±150)	337 (±165)
Plugged green	109 (±133)	365 (±138)
Control plugged	225 (±136)	251 (±138)

[a] All sample sizes were 20.

data in two comparisons weakens the P values obtained slightly, but since only two comparisons are made with each data set, the robust results obtained are not materially changed. In every case, the t test values associated with comparison of neutral and full down postures yielded similar conclusions, thus just the results for the comparison of neutral postures are mentioned.

The responses shown to self water were very different from those shown to standard *O. rusticus* water ($P = 0.0001$), as in previous studies (Hazlett, 1985a). The responses shown to ammonium were significantly different from those shown to self water ($P = 0.013$) with more time spent in neutral postures. The responses to water from animals with plugged green glands were not significantly different from the ammonia tests ($P = 0.605$), but they were significantly different from the control plugged animals ($P = 0.001$). The latter were not different from the standard disturbed *O. rusticus* tests ($P = 0.989$). Because ammonium is given from the gills in crayfish, the animals with the green gland plugged were still giving off ammonia; thus the similarity of the response to ammonia and plugged green gland conditions is to be expected. The above results strongly suggest that the disturbance chemical response is to a composite of perception of ammonia and possibly other chemicals (from the gills) and some substance or substances released from the green gland. The green gland has been identified as the source of a number of pheromones in crustacea (Gleeson, 1980, and references therein).

The results of the ablation experiments (Table 3) clearly indicate that the antennules are the site of detection of the chemical(s). When water from disturbed *O. rusticus* was introduced to animals with the antennae removed, the response was not different from that shown by intact animals ($P > 0.05$). The responses of the animals with antennules removed was significantly different from the responses of animals without antennae ($P < 0.001$) but was not different from responses shown to self water ($P > 0.10$). Antennuleless animals behaved as if there was no chemicals in the water.

The studies of the nature of the response to disturbance pheromone will be

TABLE 3. AVERAGE (±SD) NUMBER OF SECONDS DURING 8-MIN OBSERVATION SPENT BY *O. virilis* WITH ONE OR MORE PARTS IN NEUTRAL AND FULL DOWN POSTURE WHEN WATER FROM DISTURBED *O. rusticus* WAS INTRODUCED[a]

Part removed	Neutral postures	Full down posture
Antennae	343 (±102)	102 (±101)
Antennules	69 (±120)	409 (±130)
None/standard	234 (±131)	232 (±145)

[a] The test crayfish had either antennae or antennules removed two days before the test. The responses shown under "standard/none" conditions are included from Table 2 for general comparison.

considered in three parts. The first question addressed was why there is a marked delay between introduction of the chemical-containing water to an aquarium and the first responses of a recipient crayfish. Utilization of five disturbed individuals of *O. rusticus* as the source of the chemical(s) did not result in any decrease in the latency of response (mean seconds to respond were 92 and 129 for one and five source crayfish, respectively, $P = 0.193$). When water from disturbed *O. rusticus* was introduced at a threefold higher rate, there was no difference in the latency (mean seconds to respond were 139 and 157 for pulse and normal rates, respectively, $P > 0.10$). The results of both tests suggest that the latency is a function of some central process rather than a detection threshold.

The results of the tests to see how long the pheromone-induced changes in behavior lasted were inconclusive (Table 4) in the sense that even after 15 min without addition of source water there was no significant difference in the pattern of response compared to the response pattern right after cessation. The fact that there was neither a time effect or a time * treatment effect in the ANOVA shows that the patterns of behavior persist for at least 15 min. Combined with the results of the test mentioned in the previous paragraph, it would appear that the behavioral response of crayfish to disturbance chemical is slow to develop but persists for some time.

The tests comparing the responses of crayfish to other types of input when primed by disturbance chemical yielded very clear results. The latency to respond to flashes of light was significantly lower when the crayfish had detected

TABLE 4. AVERAGE (±SD) NUMBER OF SECONDS SPENT DURING 5-MIN PERIODS FOLLOWING CESSATION OF DISTURBANCE PHEROMONE BY *O. virilis* WITH ONE OR MORE PARTS IN NEUTRAL AND FULL DOWN POSTURE[a]

	Neutral postures	Full down posture
First 5 min		
Control	28 (±67)	268 (±77)
Disturbance	166 (±124)	134 (±125)
Second 5 min		
Control	41 (±71)	258 (±71)
Disturbance	201 (±93)	98 (±93)
Third 5 min		
Control	31 (±69)	268 (±64)
Disturbance	112 (±95)	178 (±105)
Time effect	$P = 0.509$	$P = 0.626$
Treatment effect	$P = 0.004$	$P = 0.008$
Time * treatment	$P = 0.325$	$P = 0.447$

[a] Ten individuals followed for 15 min each for both test and control periods.

disturbance chemical(s) (mean of 6.8 sec vs. 35.5 sec, $P = 0.038$). Similarly, the latency to respond to the input of chemicals associated with food was significantly shorter (73.3 vs. 115 sec, $P = 0.032$).

The results of the tests using other species of *Orconectes* as recipient crayfish yielded rather surprising results. For both *O. propinquus* (Table 5) and *O. rusticus* (Table 6), there was no difference in the postures shown by crayfish when water from disturbed and undisturbed crayfish was introduced. For both species, the recipient crayfish's postures indicated they were well adjusted (undisturbed) to the laboratory situation. Their lack of response to water from different sources was not all because the test individuals were too active in the laboratory situation. The one marginally significant result in all the comparisons (neutral postures for the *O. propinquus–O. propinquus* tests) was in the opposite direction to that expected. In would appear that individuals of these two species do not respond to disturbance chemicals given off by conspecific individuals.

TABLE 5. AVERAGE (±SD) NUMBER OF SECONDS DURING 8-MIN OBSERVATION SPENT BY *O. propinquus* WITH ONE OR MORE PARTS IN NEUTRAL AND FULL DOWN POSTURE WHEN WATER FROM VARIOUS SOURCES (SAMPLE SIZES IN PARENTHESES) WAS INTRODUCED[a]

	Neutral postures			Full down posture		
Source water	Undisturbed	Disturbed	*P*	Undisturbed	Disturbed	*P*
O. propinquus (18)	199 (±182)	101 (±128)	0.042	197 (±210)	183 (±186)	0.696
O. virilis (18)	155 (±162)	206 (±162)	0.257	203 (±199)	125 (±166)	0.110

[a] *P* values associated with paired *t* tests comparing responses shown to water from undisturbed and disturbed source animals.

TABLE 6. AVERAGE (±SD) NUMBER OF SECONDS DURING 8-MIN OBSERVATION SPENT BY *O. rusticus* WITH ONE OR MORE PARTS IN NEUTRAL AND FULL DOWN POSTURE WHEN WATER FROM CONSPECIFIC INDIVIDUALS WAS INTRODUCED[a]

	Neutral postures	Full down posture
Undisturbed	53 (±68)	390 (±143)
Disturbed	77 (±94)	361 (±155)
	$P = 0.364$	$P = 0.459$

[a] *P* values associated with paired *t* tests comparing responses to water from undisturbed and disturbed source animals. Sample size was 20.

DISCUSSION

Since environmental disturbances induce biochemical changes in a variety of animals (Kerambrun and Guérin, 1984) and disturbances can affect a wide variety of animals in local communities, we might expect obtaining information about disturbances by chemical means to be widespread. The responses of crayfish to water occupied by disturbed (more active) and undisturbed (less active) aquatic animals point to a very general channel of chemical communication in a very specific case. Individuals of *Orconectes virilis* respond to a chemical or chemicals given off by a wide variety of animals when the latter are disturbed (induced to a high level of activity). At the same time, other species of crayfish show no such response. The latter is particularly surprising since both *O. propinquus* and *O. rusticus* give off a chemical to which individuals of *O. virilis* respond (Hazlett, 1985a). In fact, the easily disturbed nature of *O. rusticus* and the clear responsiveness of *O. virilis* to disturbed *O. rusticus* led me to use *O. rusticus* as the preferred source species for the chemical(s) (Hazlett, 1985a, this report). *O. propinquus* does distinguish water from conspecifics vs. *O. virilus* (Tierney and Dunham, 1982) but shows no sign of distinguishing between disturbed and undisturbed animals of either species.

There are no obvious differences in the ecology of the three species of crayfish tested that correlate with the pattern of responsiveness. All three species can be found in the same stream (Capelli and Capelli, 1980), although there is evidence for both resource partitioning between *O. virilis* and *O. propinquus* (Quinn and Janssen, 1989; Rabeni, 1985) and species displacements by *O. rusticus* (Capelli and Munjal, 1982; Capelli and Magnuson, 1983). Both of these processes point toward similarities in resource utilization by the three species. *O. virilis* does have a more complex mating system (Ameyaw-Akumfi and Hazlett, 1975; Hazlett, 1983) than either *O. propinquus* (Ameyaw-Akumfi, 1976) or *O. rusticus* (Berrill and Arsenault, 1982), but this only indicated a generally higher level of behavioral complexity in *O. virilis* and does not offer ecological/evolutionary causal mechanisms. The lack of responsiveness in crayfish known to produce a chemical message does point towards a communication system that has been shaped only on the recipient side.

The wide phyletic diversity of aquatic species that can act as sources of a chemical signal and the suggestion that a waste product (ammonia) may be a part of the chemical signal also suggest a system that has been modified only in the receiver. Other crustacea can detect ammonia at concentrations similar to that used in this study (Derby and Atema, 1982a,b), but it appears that a behavioral response to such detection does not occur in *O. rusticus* and *O. propinquus*, while it does occur in *O. virilis*.

The results of the series of tests on the source of the chemical signal in

crayfish very strongly suggest there are chemicals released from the green gland of crayfish as well as ammonia from the gills that contribute to the response by recipient *O. virilis*. A chemical signal generated from two sites within a sender is somewhat unusual, although this has been reported in other aquatic organisms (Rittschof and Brown, 1986), and multiple sites are not unknown (Heschl, 1989).

All of the above brings up an important semantic question. When at least a portion of the response to a chemical signal is to a very general waste product, should it be called a pheromone? Or is the term semiochemical more appropriate? In their original definition of pheromone, Karlson and Lusher (1959) emphasized intraspecific chemical communication by secreted substances but allowed that "overlap" between closely related species could occur. Katz and Shorey (1979) also stressed the intraspecific nature of pheromonal action. When it appeared that other species in the genus *Orconectes* gave off the chemical(s) (Hazlett, 1985b), the use of the term pheromone was not overextended. However, when at least a portion of the response by individuals of *O. virilis* appears to be elicited by a waste product common to almost all aquatic animals, the use of the term pheromone seems inappropriate. The green-gland-derived portion of the response may fit the broad definition of pheromone, but the overall response may best be described as a semiochemical (Norlund and Lewis, 1976) response, since it can involve both intraspecific and interspecific communication.

Whatever this system is called, it appears that it has three interesting features. As is true with other "alarm" systems (Lawrence and Smith, 1989), there appears to be a marked delay between probable detection of the chemical(s) and initiation of a behavioral response. At the ecological level this makes sense in that short spurts of "disturbance" chemicals are probably not indicative to a receiver of a general, potentially dangerous situation while a prolonged chemical message may signal such conditions in the environment. This leads to the second behavioral feature of the response, which is its long duration. Once on alert (to potential changes of an unspecified nature), it would be to the animal's advantage to be wary for some time. The increased level of responsiveness to various types of stimulus input (the third feature of the system) further suggests that the function of the response to the disturbance semiochemical in *O. virilis* is to be ready to react to any of a variety of situations. Whether this type of response by individuals of *O. virilis* is a result of past selection or the result of individual experience can only be answered by studies of the ontogeny of the system (Rittschof et al., 1984).

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ALLELOCHEMICALS IN SOIL FROM NO-TILLAGE VERSUS CONVENTIONAL-TILLAGE WHEAT (*Triticum aestivum*) FIELDS[1]

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Abstract—Putative allelochemicals found in the soil of no-tillage and conventional-tillage wheat plots near Stillwater, Oklahoma, were obtained by a mild alkaline aqueous extraction procedure, bioassayed to determine their biological activity, purified, and analyzed with a capillary gas chromatography–mass spectrometry–data analysis system. The most significant inhibition was found in bioassays of extracts from soil collected immediately after harvest in June, July, and August. No-tillage soils produced significant inhibition during the rest of the year also. Mass spectrometry showed fatty acids as the most abundant compounds. However, when bioassayed authentic samples of the five free fatty acids showed no significant biological activity toward wheat.

Key Words—Allelochemicals, no-tillage, conventional-tillage, soils, wheat, *Triticum aestivum*, mass spectrometry, Petri-dish bioassay, fatty acids.

INTRODUCTION

Growing wheat without conventional tillage of the soil has become common in the last 25 years. There are various versions of "conservation tillage" practice, but the simplest and most common is no-tillage in which the wheat is planted directly into the residue of the past crop. Soil erosion is reduced and there are

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other benefits, but the residues may also harm the next crop. A summary of the benefits and difficulties of this practice is given by Waller et al. (1987).

Several other workers have studied allelopathy in wheat and other small grains (McCalla and Duley, 1949; Guenzi and McCalla, 1962; Norstadt and McCalla, 1963; McCalla and Haskins, 1964; Kimber, 1973; Wallace and Elliott, 1979; Wallace and Whitehand, 1980). The literature on allelopathy, including substantial amounts of work on crops and their residues, has recently been reviewed by Rice (1984) and by Putnam and Tang (1986). Elliott et al. (1984) reviewed the extensive literature on various aspects of the rhizosphere soil; allelochemicals can be released into soil as a result of root and microbial exudates and/or metabolism. They can accumulate in the rhizosphere and probably can combine with humus. Samtsevitch (1965) estimated that the dry weight of root mucilage caps deposited in the soil of wheat was similar to the dry weight of grain produced. Although the allelopathic properties of wheat have been studied at several times and places, little research has been done on accumulated allelochemicals in untilled wheat soil, and apart from our own studies (Waller et al., 1987; Thorne et al., 1990), we know of none in the southern Great Plains region of the United States.

Numerous compounds have been isolated and identified from plant residues, including those of small grains (Rice, 1984; Thompson, 1985; Putnam and Tang, 1986; Waller, 1987; Waller et al., 1987; Thorne et al., 1990). The presence of an allelochemical in plant residue does not necessarily mean that it can escape into the environment where it can affect recipient plants. Fuerst and Putman (1983) suggested that, as one of the four guidelines in the proof of allelopathy, suspected compounds must be detected in the environment around the recipient.

Using relatively gentle alkaline–aqueous methods, soils were leached from conventional-tillage (CT) and no-tillage (NT) plots. Bioassay techniques were used to survey the resulting extracts for allelopathic activity, and mass spectrometry to identify several compounds, although not ones active toward wheat.

METHODS AND MATERIALS

Sample Characteristics. Soils were taken from plots that had been under tillage treatment (CT or NT) for five years at the beginning of the study. These plots belong to the Department of Agronomy, Oklahoma State University, and are part of a long-term study of tillage practices. The soil was Easpur fine sandy loam, fine-loamy, mixed Thermic Fluventic Haplustoll (Oklahoma Department of Agriculture, 1987). The CT plots were moldboard-plowed 15–20 cm deep, and disked, shortly after harvest. Thus, essentially all residue was buried for about four months. NT plots were replanted with no disturbance other than the

planting itself, so that both standing stubble and fallen straw, leaves, and chaff were present. Soil samples were collected monthly by bulking 8–10 cores per plot from two plots of each type. Cores were of the upper 3 cm of soil, plant residue having been brushed aside. Samples were taken from January 1986 through February 1987 and were placed in jars, frozen in the field with Dry Ice, and stored frozen at −18°C until used. The pH of in situ soil in 1986 was 5.0 in NT and 5.8 in CT plots. Rainfall for the period of February 1986 through January 1987 was 82 cm.

Extraction and Purification. Allelochemicals were extracted by making a slurry of 400 g freshly thawed soil and 800 ml distilled water and adjusting the pH to 8.0 ± 0.2 with 1 M NaOH (approximately 8 ml for CT and 12 ml for NT soils). This mixture was shaken for 2 hr at 5–10°C, allowed to stand for 2 hr at 5–10°C, and then filtered through glass wool. Material trapped by the filter was discarded and the filtrate centrifuged for 25 min at 15×10^3 rpm (Sorvall Superspeed RC2-B). The supernatant fluid was microfiltered through a 0.45-μm glass fiber filter (MSI MAGNA, Millipore Corp.) and divided into three portions; 10 ml were used for an immediate bioassay, 10 ml for a final pH test (usually 7.4–7.6 for CT and 7.1–7.3 for NT) and 400 ml was lyophilized, weighed, and reserved for later use. The procedure was conducted at least twice for each monthly set of soil samples.

In using the lyophilized residue for bioassay, an amount of material equivalent to 5 g of soil was extracted with three 10-ml portions of hot methanol (MeOH extract). A second extract was made in the same manner using material from 25 g soil (concentrated MeOH extract). The methanol solutions were vacuum-filtered through Whatman No. 1 filter paper, evaporated to dryness in a hood at room temperature, and redissolved in methanol. Frequently it was necessary to microfilter the extract again due to presence of insoluble material in the dried residue, possibly caused by oxidation and polymerization during the process.

Analysis was begun by extracting 150 mg of each month's lyophilized residue three times with 25-ml portions of hot methanol. The combined extracts were suction-filtered through Whatman No. 1, the methanol evaporated at room temperature, and the remaining residue reextracted three times with 1-ml portions of hot methanol. The extracts were combined, further purified by microfiltering through a 0.45-μm glass fiber or nylon membrane filter, then evaporated to dryness under nitrogen.

Bioassay. Each bioassay test or control used four, 100 × 15-mm glass Petri dishes containing two sheets of 7.5-cm Whatman No. 1 filter paper. Ten seeds per dish of Pioneer brand 2157 wheat were placed between the filter paper disks. Two milliliters of the aqueous test solution (adjusted to pH 7.0 ± 0.2) was added to the dish and 2 ml distilled water was used in the control dishes. For solutions with organic solvent and solvent control, they were applied to the

filter paper and allowed to dry before seeds were placed in the dishes. Two milliliters of distilled water were placed in each dish, a sheet of plastic film stretched over it to retard evaporation, and the cover replaced. After a 72-hr incubation at 20°C in darkness, the length of each seedling root and shoot was measured.

Bioassays also were carried out on commercial (>99% purity) fatty acids. Ether solutions (1.0 mM) of myristic, palmitic, heptadecanoic, stearic, and eicosanoic acids were prepared and tested separately, and in all cases the total concentration was held at 1.0 mM. In addition, a bioassay of all five fatty acids together was performed at a total concentration of 1.0 and 5.0 mM.

Statistical Analaysis of Data. The year's bioassay data were subject to a two-way analysis of variance (ANOVA) to determine the statistical significance of differences attributable to the two tillage treatments versus the distilled water control, attributable to the date of soil collection, and attributable to treatment × date interactions. Separate one-way ANOVAs were performed on each month's data to determine significance of tillage treatment effects, and Tukey's studentized range test was employed to determine which treatments (CT, NT, and control) differed significantly from which others. Moreover, the correlation coefficient between the weight of the lyophilized solids from an extract and the growth of bioassay seedlings in that extract was calculated, to indicate whether the effects of the extracts were due to qualitative or quantitative chemical differences.

Low-Resolution Mass Spectrometry. To improve resolution and separation of the compounds of interest, acids were converted to the methyl esters. Accordingly, 1 ml of diazomethane was added to the dried residue and the container was shaken for 5 min or until the yellow color disappeared, indicating completion of the reaction. As an internal standard, 1.0 mg of caffeine was added to each sample. Any excess diazomethane and its solvent, ethyl ether, were evaporated under nitrogen. The diazomethane was synthesized by the method of Ruehle et al. (1979).

Low-resolution mass spectra of the diazomethane-treated samples were obtained using an LKB-2091 (LKB Produckter, Bromma, Sweden) capillary gas chromatograph—mass spectrometer–data analysis system (CGC-MS-DAS) (McGown and Waller, 1986). These devices were linked so that the chromatographic column was directly connected to the ion source of the mass spectrometer and data output went directly to the data-analysis computer. The column was 30 m × 0.32 mm DW-5 (J & W Scientific, Folsom, California), and a flow rate of 2–3 ml He/min was used. Up to 2.0 μl of sample in methanol was injected with a 1:4 split at 65°C. The temperature was programmed to rise (after 4 min) 10°C/min until 300°C was reached, and then to remain there for 10 min.

The mass spectrometer conditions were: injector temperature 275°C, separator temperature 275°C, initial eV 21, scan eV 70, box current 15–20 μA,

accelerating voltage 3.2–3.4 V, filament current 3.3 A, trap current 80–100 A, multiplier voltage 500 mV, and source temperature 265°C.

Identification of spectra was done both visually and by automated [IBM AT microcomputer with Technivent (St. Louis, Missouri) programming system] means. Comparison was with known spectra given in the Eight Peak Index of Mass Spectra (1983), by Waller (1972), and by Waller and Dermer (1980).

High Resolution Mass Spectrometry. Confirmation of the identity (by formula: C, H, O, N) of each ester mentioned in this paper was done using a model 70-SE spectrometer (CGC-MS-DAS) (VG Instruments, Manchester, United Kingdom).

RESULTS

Yield of Extracts. No-till extracts generally yielded more lyophylized residue (802 ± 58 mg) than CT (481 ± 54 mg) from 400 ml of the crude soil extracts. They ranged in ratio from 1.0 to 3.6 and averaged 1.67 (NT:CT).

Bioasssay of Aqueous Extracts. Analysis of variance on bioassays of direct aqueous extracts revealed highly significant variation ($p < 0.0001$) in root lengths attributable to the main effects of treatment and date, and also the treatment × date interaction. On an annual basis, seedling roots were significantly longer ($p < 0.01$, Tukey's test) in the CT extracts than in the distilled water control and significantly shorter in the NT extracts than in the control (Table 1). Trends were the same for shoot lengths, but not statistically significant. Because of the significant treatment × date interaction, these annual results are less revealing than the month-by-month comparisons.

TABLE 1. ANNUAL MEAN ROOT AND SHOOT LENGTHS (mm)[a]

Treatment[b]	Aqueous extract	MeOH extract	Conc. MeOH extract
Root			
DW	27.56a	26.29a	26.29a
CT	28.83b	25.19b	24.49b
NT	26.04c	24.46c	23.09c
Shoot			
DW	7.42a	7.28a	7.29a
CT	7.47a	7.35a	7.54a
NT	6.97a	6.85b	6.84a

[a] Means with the same letter in a given column are not significantly different ($P > 0.05$) by Tukey's studentized range test.
[b] DW = distilled water; CT = conventional-tillage; NT = no tillage.

Figure 1 shows monthly means for roots and shoots, expressed as a percentage of the distilled water control. This is a useful method of presentation because both stimulation (values > 100%) and inhibition (< 100%) are clearly expressed; however, it is not amenable to presentations of statistical significance levels because each point on the graph represents a quotient of two means.

Results of Tukey's test will be described below; all differences noted were statistically significant with $P < 0.05$ (for full results see Cast, 1987). Roots of seedlings exposed to NT soil extracts were significantly inhibited relative to the control in February, June, July, and August. NT extracts stimulated root growth relative to the control in May, but in no cases were NT roots significantly longer than CT. Root growth in CT extracts was stimulated relative to control in March, April, May, and June but inhibited relative to control in July and August. Shoot growth showed similar patterns, although fewer comparisons

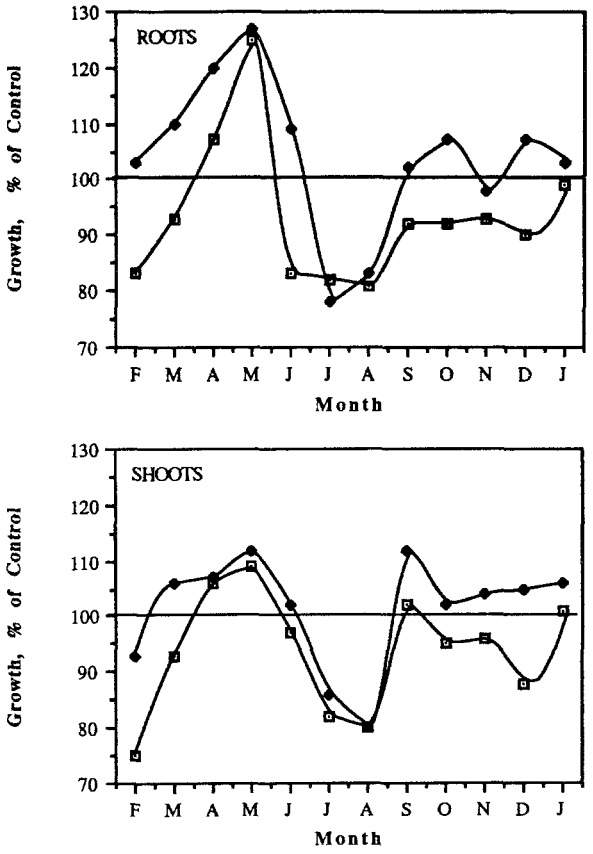


FIG. 1. Growth as a percentage of control from bioassay of direct aqueous extract of NT (⊡) and CT(♦) soils from February 1986 to January 1987.

were statistically significant. NT treatments were inhibitory relative to control in February, August, and December. CT was stimulatory in May and September, but inhibitory in July and August.

Bioassay of Methanol Extracts. An important point of difference between the methanol and aqueous bioassays was that significant stimulation of root or shoot growth, while common in aqueous extracts, was observed only once in methanol extracts (shoots assayed with December MeOH extract). On an annual basis (Table 1), the distilled water control had significantly longer roots than either extract. In general, NT extracts were broadly inhibitory; CT extracts were inhibitory when prepared from soils collected in the late summer months.

Figure 2 shows the monthly means (as percentage of control) for the bioassays of methanol-soluble fractions of the lyophilized aqueous extracts, at

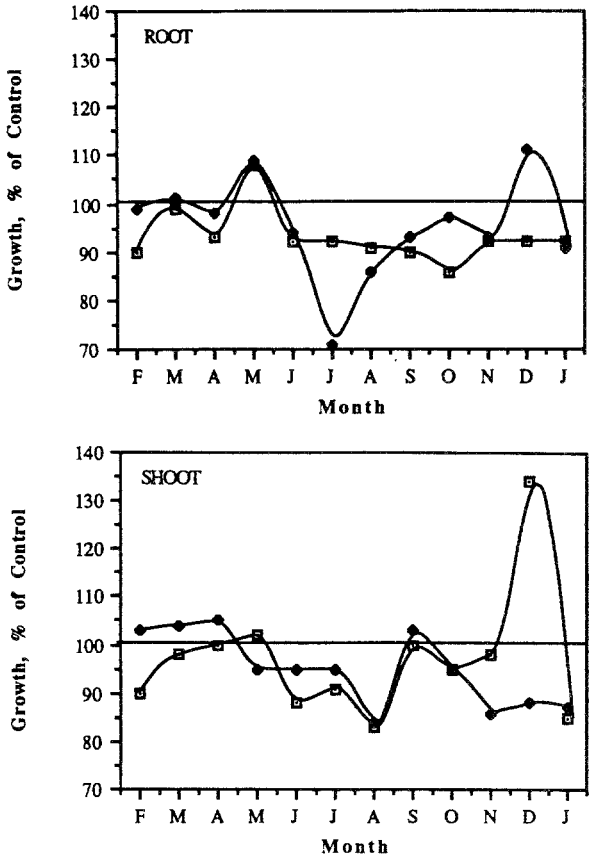


FIG. 2. Growth as a percentage of control from bioassay of methanol extract of lyophilized residue at approximately the same concentration as the aqueous extract of NT (⊡) and CT (♦) soils from February 1986 to January 1987.

approximately the same concentrations as the aqueous. Figure 3 shows the same information for the concentrated MeOH extract. For the MeOH extracts of NT soils, roots were significantly inhibited relative to control by soils collected in every month from June through November, and shoots were inhibited by extracts from January, June, July, and August. CT extracts were inhibitory to roots in July, August, and September soils only, and to shoots only in January and August. Inhibition was stronger, as might be expected, in the concentrated MeOH extracts. NT soils from February, April, June, and August through December were all inhibitory to roots, and those from June, August, September, and October were inhibitory to shoots. Significant inhibition by CT extracts

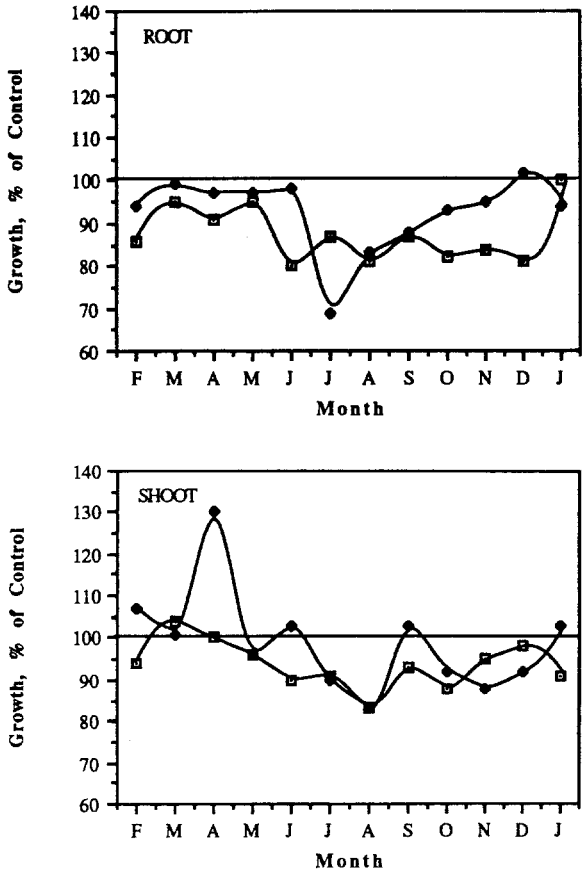


FIG. 3. Growth as a percentage of control from bioassay of methanol extract of lyophilized residue at approximately three times the concentration as the aqueous extract of NT (⊡) and CT (♦) soils from February 1985 to January 1986.

was seen in July–October for roots, and in July, August, October, and December for shoots.

A puzzling feature of the methanol bioassay was that July CT extracts produced less root growth than NT extracts, and November CT extracts produced less shoot growth than NT extracts. These statistically significant differences were observed in both the MeOH and concentrated MeOH preparations. This result is unexpected and unexplained.

Correlation Analysis. In no cases were significant correlations observed between the quantity of lyophilized residue recovered from aqueous extracts and the growth of seedlings in bioassay. This implies that inhibition or stimulation of root growth was caused by qualitative differences among the extracts rather than quantitative dosage effects. These data must be interpreted with caution, however, because the lyophilized residues consisted primarily of inorganic materials, and it is still possible that organic constituents present in small quantities could act in a dosage-dependent manner.

Chemical Analysis. The two instruments yielded similar results except that the VG 70-SE provided high resolution of the individual compounds. The methyl esters of fatty acids were found to dominate the methanol fraction of soil throughout the year. Figures 4 and 5 show the reconstructed total ion current chromatogram with some identification of individual compounds for the NT (Figure 4) and CT (Figure 5) samples for June 1986. There were more peaks in the NT (26 compounds) than in the CT chromatograms (20), indicating that some of the unidentified compounds were allelochemicals that would require

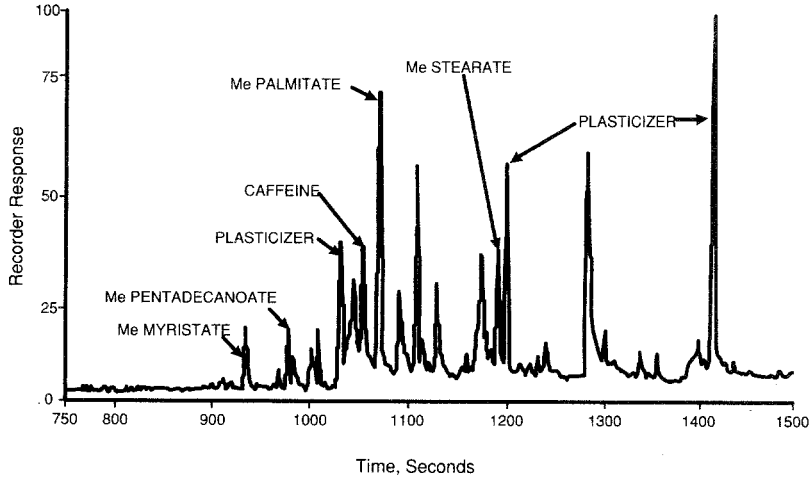


FIG. 4. Reconstruction total ion current chromatogram of the methanolic fraction from lyophylization of NT soil extract (LKB-2091 CGC-MS-DA): Peaks represent compounds; identified peaks are labeled. Soil sample: June 1986.

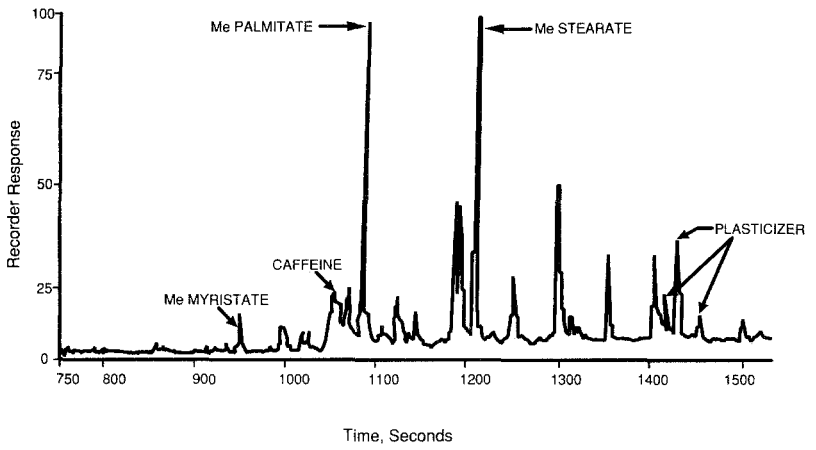


FIG. 5. Reconstructed total ion current chromatogram of the methanolic fraction from lyophylization of CT soil extract (LKB-2091 GCC-MS-DA): Peaks represent compounds, and identified peaks are labeled. Soil sample: June 1986.

further derivatization before they could be identified. Caffeine peaks represent the internal standard, and phthalate plasticizers constituted artifactual compounds obtained from the soil (Waller et al., 1987). In all months and treatments, the most prevalent fatty acids (seen as the methyl esters) were palmitic and stearic acids; shorter-chain fatty acids such as myristic acid and pentadecanoic acid were present in much lower concentrations.

Fatty Acid Bioassay: Fatty acids were regarded as possible allelochemicals in the soil of wheat systems. Not only were these the main organic compounds in the extracts, but other researchers have considered fatty acids as possible allelochemicals. Much of the previous research focused upon volatile, short-chain fatty acids (Stevenson, 1967; Lynch 1977; Tang and Waiss, 1978; Wallace and Elliott, 1979; Wallace and Whitehand, 1982), but some long-chain ones have been considered (Spoehr et al., 1949). Alsaadawi et al. (1983) investigated allelopathic interaction of nine fatty acids in decomposing residues of *Polygonum aviculare* (myristic, palmitic, linolelaidic, oleic, stearic, arachidic, 11,14-eicosadienoic, heneicosanoic, and behenic acids), and they found all except myristic and heneicosanoic acids in soil under such residues. Sodium salts of all of these fatty acids inhibited seedling growth of Bermuda grass and also some test strains of *Azotobacter* and *Rhizobium* at a concentration of 5 ppm. Waller et al. (1984, 1985, 1987) found exhaustive steam distillation of wheat soil (Tillman, clayloam fine, mixed, Thermic Typic Haplustolls) where wheat had been grown for the past decade to yield *n*-saturated fatty acids from C_4 to C_{18} as well as other compounds; the Petri-dish bioassay results showed different amounts of inhibition in the fractions (neutral, acidic, and alkaline)

TABLE 2. RESULT OF BIOASSAY WITH WHEAT OF PURE FATTY ACIDS AT 1.0 mM

Fatty acid	Growth (% of control)	
	Root	Shoot
Myristic	107.1	94.4
Palmitic	89.9	81.3
Heptadecanoic	112.9	98.1
Stearic	97.6	85.0
Eicosanoic	100.9	96.7

with neutral and acidic being less so that the basic fraction. As expected, most fatty acids were found in the acidic fraction of steam distillation.

Since the predominant compounds in the methanol fraction were free fatty acids, the bioassay results of authentic ones are shown in Table 2. For those fatty acids assayed at 1.0 mM, no statistically significant inhibition or stimulation was noted. When five fatty acids were mixed (1 mM and 5 mM concentration) and bioassayed to test for synergistic effects, no significant effect was seen. Based on these results, as well as those from exhaustive steam distillation of wheat soil, it was our finding that the long-chain fatty acids do not have a significant allelochemical action toward wheat.

DISCUSSION

Stimulation of seedling growth in bioassays was found in aqueous extracts but seldom in methanol extracts. This suggests that stimulation may have been a consequence of water-soluble compounds leached out of the soil samples.

NT extracts were broadly inhibitory, with roots showing this inhibition most clearly. Significant inhibition by at least one of the NT extracts was found in every month except March and May. CT extracts were most commonly observed to be inhibitory in soils collected from July through September. This is the period of time following wheat harvest when straw and stubble are still standing or have been recently plowed under, and conditions for both NT and CT would be similar. This observation leads further support to the hypothesis that allelochemicals in wheat soils arise from wheat residue.

Wheat field soils collected at monthly intervals for one year and analyzed by MS-CGC-DAS showed that stearic and palmitic acids were the major compounds present in each month. Other data (Waller et al., 1987) support the consistent presence of stearic and palmitic acids in wheat field soils in Oklahoma. There was a definite tendency toward greater autotoxicity in the no-tillage soil compared to conventional tillage; however, it was clear that the long-

chain fatty acids did not produce this effect because they had little effect on germination and early growth of wheat. Our data suggest that there are one or more organic allelochemicals present in wheat soils, especially untilled ones, but we have not identified them.

Microbial breakdown of wheat residue is slowed by no-till practice (Lynch, 1987; Elliott and Cheng, 1987). It seems likely that the allelochemicals responsible for the inhibition are microbial products that accumulate more in no-till than in conventionally-tilled soil because it is not disturbed and dispersed by cultivation. We recognize that environmental conditions, especially temperature, moisture, and soil texture, greatly influence microbial activity. Thus, it further seems likely that there will be large variations in allelochemical content of the soil from place to place and year to year. Under some conditions, this could be a substantial problem to wheat growers. Research is continuing on the nature and source of the allelochemicals and the conditions influencing their production.

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EFFECT OF STEAM DISTILLATE EXTRACTS OF SELECTED RESISTANT CULTIVATED AND WILD RICES ON BEHAVIOR OF LEAFFOLDER *Cnaphalocrocis medinalis* (Guenée) (LEPIDOPTERA: PYRALIDAE)

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Abstract—Rice plant volatiles extracted as steam distillates significantly affected the behavior of the rice leaffolder, *Cnaphalocrocis medinalis* (Guenée). Leaffolder moths laid significantly fewer eggs on TN1 rice plants treated with extracts of resistant wild species of rice, *Oryza officinalis* and *Oryza punctata*, than on TN1 plants treated with extracts of resistant cultivated rices. Extracts of *O. officinalis* and *O. punctata* adversely affected egg hatchability. *O. officinalis* and *O. punctata* extracts were more toxic to first-instar larvae than extracts of other resistant varieties. Leaf area consumed by the larvae was reduced on TN1 plants treated with *O. officinalis* and *O. punctata* extracts compared with that on plants treated with extracts of resistant cultivated rice varieties.

Key Words—Rice leaffolder, *Cnaphalocrocis medinalis*, Lepidoptera, Pyralidae, *Oryza officinalis*, *Oryza punctata*, *Oryza sativa*, rice plant volatiles, egg hatchability, egg laying.

INTRODUCTION

The rice leaffolder, *Cnaphalocrocis medinalis*, is distributed throughout South and Southeast Asia, where its importance has increased in recent years. The larva rolls the leaf by tying silk to the margins and feeds within the rolled leaf, removing the green layer. Heavily damaged fields turn brown and appear scorched (Heinrichs et al., 1985). Resistance to *C. medinalis* involves the moths'

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nonpreference for oviposition, reduced larval survival (Medina and Tryon, 1986) and reduced feeding rate (Villanueva and Khan, 1988).

Biochemical factors are far more important in imparting resistance to insects as plant chemicals affect insect behavior and physiology in a number of ways (Saxena, 1986). Many authors have demonstrated the role of rice plant volatiles in conferring resistance to the brown planthopper, *Nilaparvata lugens* (Stal) (Saxena and Okech, 1985); the green leafhopper, *Nephotettix virescens* (Distant) (Khan and Saxena, 1985); and the whitebacked planthopper, *Sogatella furcifera* (Harvath) (Khan and Saxena, 1986). The biochemical basis of susceptibility or resistance of rice varieties to striped stem borer, *Chilo suppressalis*, also has been studied at the International Rice Research Institute recently (Saxena, 1986). According to Saxena and Okech (1985), rice plant volatiles are odoriferous and volatile in nature and are important in determining susceptibility or resistance to pests.

In the present study, we investigated the effect of plant volatiles obtained as steam distillate extracts from selected resistant cultivated rice varieties and wild species of rice on the behavior of *C. medinalis*.

METHODS AND MATERIALS

Steam distillate extracts of 50-day-old plants of cultivated rices Ptb 33, Rathu Heenati, and Swarnalata, and wild rices *Oryza officinalis* and *O. punctata* were bioassayed against greenhouse-reared *C. medinalis*.

Steam Distillation and Extraction of Rice Plant Volatiles. Leaves of 50-day-old plants of leaffolder-resistant cultivated and wild rices grown in an insectproof screen house were harvested and ground with an electric grinder. The steam distillation and extraction were done following the method of Saxena and Okech (1985). A 200-g ground sample was steam distilled for 3-hr, during which time approximately 900 ml of distillate were collected. The distillate was extracted with diethyl ether (300 ml of distillate–100 ml diethyl ether) by thoroughly shaking a mixture of the two together in a separating funnel for 5 min. Diethyl ether extracted the essential oils and other volatiles, and the ether mixture settled above the water layer in the funnel. If an emulsion was formed while mixing, a few crystals of sodium sulfate were added to break the emulsion. The water layer was discarded while the ether extract was pooled in a 500-ml beaker, in which 50 g of anhydrous sodium sulfate were added. The resultant mixture was kept inside a fume hood to evaporate excess ether until the remaining volume was approximately 25 ml. The beaker then was covered with aluminum foil and held overnight to allow sodium sulfate to absorb traces of water from the extract. The extract was evaporated further to 10 ml and decanted into a

weighed glass vial, which was then covered with perforated aluminum foil and placed inside a desiccator. Ether was evaporated under vacuum, leaving behind a yellow oily residue. The vial was reweighed, sealed with nitrogen, and kept at −10°C. The yellow oily residue was diluted in acetone–water (4:1) and tested at 2000 ppm.

Effect of Steam Distillate Extracts of Resistant Cultivated and Wild Rices on Ovipositional Behavior of C. medinalis. Using a quick-spray atomizer, a single tiller of 30-day-old susceptible TN1 plants grown in 16-cm-diameter pots were sprayed with 1 ml of acetone–water solutions of extracts of resistant cultivated and wild rices at a rate of 2000 ppm. Control plants were untreated or treated with acetone. Each potted plant was covered with a Mylar cage (90 cm height × 10 cm diam.) and infested with five pairs of freshly emerged *C. medinalis* adults and the number of eggs laid per treatment were counted three days after release. The experiment was conducted in a randomized complete block design and replicated five times.

Effect of Steam Distillate Extracts of Resistant Cultivated and Wild Rices on Egg Hatchability of C. medinalis. Fifty freshly emerged adults of *C. medinalis* were allowed to oviposit for a day on 30-day-old TN1 plants enclosed in a fiberglass net. A 5-cm leaf piece bearing freshly laid eggs in batches of 20 were dipped in a 2000 ppm solution of the extract and excess liquid drained. The eggs were incubated in Petri dishes (10 cm diam.) lined with moistened filter paper, and the number of hatched and unhatched eggs were recorded. Each treatment was replicated five times and arranged in a randomized complete block design.

Toxicity of Steam Distillate Extracts to First-Instar Larvae. Ten 6-cm-leaf pieces of the susceptible TN1 rice plants were dipped separately in a 2000 ppm solution of extracts of resistant cultivated and wild rices and placed in Petri dishes (10 cm diam.) lined with moistened filter paper. After the acetone had evaporated, 10 first-instar larvae were released on each of the treated or control leaf pieces. Each treatment was replicated five times and arranged in a randomized complete block design. Larval mortality was recorded after 72 hr.

Effect of Steam Distillate Extracts of Resistant Cultivated and Wild Rices on Leaf Area Consumed by First-Instar Larvae. Five-centimeter-long leaves cut from the middle portion of 30-day-old susceptible TN1 plants were dipped in 2000 ppm solutions of extracts of resistant cultivated and wild rices and placed inside Petri dishes (10 cm diam.) lined with moistened filter paper. Ten 1-day-old first-instar larvae were placed in each Petri dish, and the dishes were arranged in a randomized complete block design replicated five times. After 48 hr, the leaf area consumed was measured using an automatic leaf area meter (Licor 3000 area meter).

RESULTS

Effect of Steam Distillate Extracts of Resistant Cultivated and Wild Rices on Ovipositional Behavior of C. medinalis. There were significant differences in the ovipositional behavior of *C. medinalis* when the adults were caged on TN1 plants treated with extracts of resistant cultivated and wild rices (Table 1). Extracts of *O. officinalis* and *O. punctata* inhibited oviposition of leaffolder moths: four to six times fewer eggs were laid on extract-treated plants than an acetone-treated and untreated plants. Significantly fewer eggs were laid on TN1 plants treated with extracts of Ptb 33, Rathu Heenati, and Swarnalata but more than on plants treated with extracts of wild rices.

Effect of Steam Distillate Extracts of Cultivated and Wild Rices on Egg Hatchability. Applications of extracts of resistant cultivated and wild rices on eggs of *C. medinalis* significantly reduced egg hatchability, but application of acetone had no effect (Table 1). The percentage of unhatched eggs ranged from 66% to 78% when the eggs were treated with *O. punctata* and *O. officinalis* extracts and was significantly higher than the eggs treated with extracts of Ptb 33, Rathu Heenati, and Swarnalata.

Toxicity of Steam Distillate Extracts to First-Instar Larvae. There were significant differences in toxicity among extracts of resistant cultivated rices, and extracts of *O. officinalis* and *O. punctata* were extremely toxic to first-instar larvae, causing 86–88% mortality of larvae, respectively (Table 1). Extracts of

TABLE 1. EFFECT OF STEAM DISTILLATE EXTRACTS OF RESISTANT CULTIVATED AND WILD RICES ON *C. medinalis* OVIPOSITIONAL BEHAVIOR, EGG HATCHABILITY, LARVAL MORTALITY, AND LEAF AREA CONSUMPTION TESTED ON SUSCEPTIBLE TN1 RICE VARIETY[a]

Treatment	Number of eggs laid	Unhatched eggs (%)	Larval mortality (%)	Leaf area consumed (cm^2)
None	232a	0d	0d	1.160a
Acetone	227a	2d	4c	1.150a
2000 ppm Ptb33	129b	46c	44b	0.786b
2000 ppm Rathu Heenati	135b	40c	40b	0.794b
2000 ppm Swarnalata	132b	44c	42b	0.766b
2000 ppm *O. officinalis*	42d	78a	88a	0.670c
2000 ppm *O. punctata*	59c	66b	86a	0.696c

[a]Mean of five replications. Means followed by a common letter are not significantly different at the 5% level by Duncan's multiple-range test.

Ptb 33, Rathu Heenati, and Swarnalata also caused high mortality of larvae but less than on TN1 leaves treated with extracts of *O. Officinalis* and *O. punctata*.

Effect of Steam Distillate Extract of Cultivated and Wild Rices on Leaf Area Consumed by Larvae. Application of steam distillate extracts inhibited feeding by larvae of leaffolder (Table 1). Leaf area consumed by the larvae on *O. officinalis*- and *O. punctata*-treated leaves was significantly less than on TN1 leaves treated with extracts of Ptb 33, Rathu Heenati, and Swarnalata, but larvae consumed significantly larger leaf areas of acetone-treated and untreated TN1 leaves.

DISCUSSION

The present study elucidated the role of rice plant volatiles as allelochemics (Whittaker and Feeny, 1971) involved in leaffolder–rice plant interactions. Application of steam distillate extract of resistant wild species of rice inhibited oviposition by *C. medinalis* and significantly fewer eggs hatched on *O. officinalis* extract-treated TN1 plants. Adverse effects of extracts of the wild species of rice *O. brachyantha* on egg hatchability of *C. medinalis* was reported earlier by Medina and Tryon (1986).

Extracts of *O. officinalis* and *O. punctata* were more toxic to first-instar larvae than extracts of cultivated resistant varieties. Medina and Tryon (1986) also demonstrated that extract of wild rice, *O. brachyantha*, was significantly more toxic to first-instar larvae of *C. medinalis* than extracts of cultivated varieties. Leaf area consumed by *C. medinalis* larvae decreased significantly on susceptible TN1 plants treated with extracts of *O. officinalis* and *O. punctata*. According to Villanueva and Khan (1988), leaf area consumed by larvae of *C. medinalis* was significantly less on *O. officinalis* and *O. punctata*.

Among the steam distillate extracts tested, extracts of wild rices *O. officinalis* and *O. punctata* conferred the highest degree of resistance. Adverse effects of extracts of *O. officinalis* and *O. punctata* on the ovipositional behavior, egg hatchability, toxicity to first-instar larvae, and leaf area consumed, as compared to extracts of resistance cultivated varieties, evidently suggest that steam distillate extracts of wild species of rice may have higher concentrations of allomones (defense chemicals). Alternatively *O. officinalis* and *O. punctata* may have new genes that regulate the synthesis of additional chemicals conferring extremely high levels of resistance of *C. medinalis*. Evidently plant volatiles of *O. officinalis* and *O. punctata* have distinct effects on insect responses and can limit insect establishment. *O. officinalis* and *O. punctata* could be used widely as possible donors in hybridization programs to incorporate resistance to major pests.

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GROWTH INHIBITION OF *Musca domestica* L. AND *Culex quinquefasciatus* (SAY) BY (−)-3-EPICARYOPTIN ISOLATED FROM LEAVES OF *Clerodendron inerme* (GAERTN) (VERBENACEAE)

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Abstract—The petroleum ether extract of *Clerodendron inerme* leaves afforded a compound that matched the clerodane compound (−)-3-epicaryoptin in physical and spectral characteristics. The test compound inhibited the development of *Musca domestica* and *Culex quinquefasciatus* larvae. First- and third-instar fly larvae reared on 3-epicaryoptin-treated diet pupariated later and earlier than their respective controls and adult emergence from puparia was inhibited. Apart from larval mortality, exposure of fourth-instar mosquito larvae to 3-epicaryoptin resulted in death at larval–pupal molt and pupal–adult eclosion, indicating inhibition of the molting process.

Key Words—(−)-3-Epicaryoptin, insect growth inhibition, *Clerodendron inerme*, *Musca domestica*, *Culex quinquefasciatus*, Diptera, Muscidae, Culicidae, plant–insect interaction.

INTRODUCTION

A number of bitter diterpenoid compounds having a clerodane skeleton isolated from various *Clerodendron* spp. have been shown to inhibit feeding of polyphagous insects (Hosozawa et al., 1974a, b; Kato et al., 1972). Leaves of *Clerodendron* spp. mixed in housefly larval diet reduced puparial weights and inhibited adult emergence (Ahmed et al., 1981). There is no report, however, on the active compound(s) from *Clerodendron* spp. responsible for growth inhibition of insects; hence the present investigation was undertaken.

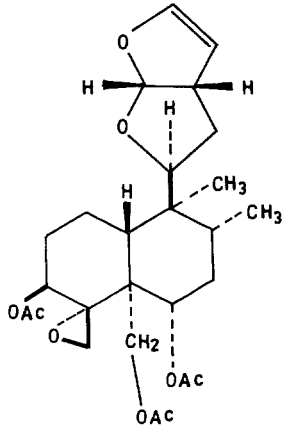
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METHODS AND MATERIALS

Isolation of Active Compound. Air-dried leaves of *Clerodendron inerme* powdered to 60 mesh were extracted in cold with petroleum ether (40–60°C) over three days with occasional stirring. The extract was filtered, evaporated in vacuo (>40°C), and the residue redissolved in petroleum ether. The soluble portion was chromatographed over silica gel and eluted successively with solvents of increasing polarity, namely, petroleum ether (40–60°C), benzene, and benzene–ether. The benzene–ether fractions were pooled and rechromatographed. The fractions were monitored by TLC (38 benzene + 62 ether) and bioassayed using third-instar housefly larvae as described later. The major compound showing maximum activity was obtained in 0.01–0.02% yield.

Structure Elucidation of Active Compound. The compound had a mp 153–155°C; $(\alpha)_D = -72°$ (c, 0.9, $CHCl_3$); Mass: *(m/z)* 492(M), 267, 249, 232, 219, 207, 202, 187, 172, etc. Its NMR spectrum (90 MHz, $CDCl_3$, TMS) showed signals for one tertiary methyl, one secondary methyl, and three acetate residues besides the signals due to a primary carbinol, an epoxymethylene, and the other characteristic signals observed for the compounds containing furofuran rings. On the whole it matched well with the NMR signals reported for 3-epicaryoptin (Hosozawa et al., 1974) isolated from *C. calamitosum*, $C_{26}H_{36}O_9$ (mol wt 492) (1). Our attempts to get an authentic specimen of I (Scheme 1) for IR spectral comparison were unsuccessful. Its IR spectrum (KBr) is given below.

The crystal structure of 3-epicaryoptin and hence its absolute configuration has been determined by X-ray crystallographic studies (Rogers et al., 1979),



(I)

SCHEME 1.

and the isolation of 3-epicaryoptin from *C. inerme* in 0.2% yield has been reported [mp 165°C $(\alpha)_D = -63.6°$(c, 1.8, $CHCl_3$). Thus the active principle closely resembled (−)-3-epicaryoptin in its physical and spectral properties.

Test Insects. Houseflies, *Musca domestica* L., and mosquitoes, *Culex quinquefasciatus* (Say), were drawn from laboratory cultures maintained for several years without exposure to insecticides.

Housefly Larval Feeding. The larval rearing medium was the same as described previously (Ahmed et al., 1981). For the assay, the required quantity of the test material dissolved in 8 ml acetone was pipetted over 6 g flocked filter paper in a 250-ml beaker. Solution was pipetted 1 ml at a time and the paper stirred continously to obtain an uniform application. For the control, 8 ml of acetone was used alone. The solvent was allowed to evaporate over 2 hr under a gentle stream of air. The liquid portion of the medium, consisting of whole milk powder (2.7 g), corn starch (2.7 g), and dried yeast (0.6 g) in 26 ml distilled water, was worked into the paper fraction by stirring with a glass rod. The prepared medium was divided into three tubes (25 × 100 mm) and covered with muslin. When four replicates were used, 13 g (dry wt) of diet was prepared. Ten eggs washed three times in sterile water or ten third-instar larvae were used per replicate. Treatment concentrations were calculated based on the total weight of the dry components of the diet. Third-instar larvae were starved by releasing them into flocked paper moistened with 2.1 ml distilled water/g paper. The number pupariating was observed every 4 hr.

Housefly Molting Hormone Assay. For this assay, 0.2–1.0 μl of 25% aqueous acetone solutions of the test compound were injected into fly larvae ligated according to Kaplanis et al. (1966). Results were based on at least five surviving specimens per treatment and repeated again: 0.2, 0.7, and 1.0 μg were injected per larva.

Mosquitoes. Larvae of fourth-instar *C. quinquefasciatus* were staged based on the truncate apex of the eye and air trumpets. The truncate apex of the eye of late fourth-instar larva was drawn out twice that of the mid-fourth-instar larva. The latter were at least 24 hr from pupation; the former 15 hr. With the first appearance of the air trumpets, pupation occurred 5 hr later. The pupae were staged according to Spielman and Skaff (1967). Individuals dying at the late pharate (PA_2) and the emerging adult (A_1) stages (Spielman and Skaff 1967) were combined and represented in the present study as PA_2. Those dead at the larval–pupal molt (L-P) were pupae with the larval cuticle attached at the cephalic region.

For the assay, up to 0.2 ml of acetone solutions of the test compound were pipetted over 50 ml distilled water in 50-ml beakers. For the controls, 0.2 ml acetone alone was used. Two hours after treatment, 25 larvae of known age were introduced into each beaker. A pinch of whole milk powder was added and fresh additions made as required. Dead specimens were observed under a

binocular microscope and the stage recorded. Emerging adults were allowed to escape.

All experiments were done at 28 ± 1°C. Percentages were subjected to angular transformations and analyzed by Duncan's multiple-range test.

RESULTS

The active compound was identified by its physical and spectroscopic characteristics as (−)-3-epicaryoptin, a clerodane diterpene belonging to the class of compounds commonly present in *Clerodendron* spp.

Tables 1 and 2 give the effect of 3-epicaryoptin on the development of first- and third-instar fly larvae, respectively. With the third-instar larvae, there was a decrease in puparial weight (Table 2), and the puparia obtained from 20 and 40 ppm treatments weighed less than the third-instar larvae used. With the first-instar larvae, there was no significant difference in the weights of puparia except at 10 ppm (Table 1). It was observed that third-instar larvae fed on the treated medium always pupariated a few hours earlier than the controls, while the first-instar larvae pupariated later.

To investigate the opposite effects on timing of pupariation of first- and third-

TABLE 1. DEVELOPMENT OF FIRST-INSTAR *Musca domestica* LARVA ON 3-EPICARYOPTIN-TREATED DIET[a]

Conc. (ppm)	Weight of puparia (mg)	Pupariation (%)	Emergence (%)[b]
0.0	22.40a	80.0a (66.91)	93.8a (79.65)
1.25	21.51a	50.0a (45.07)	78.9b (62.76)
2.50	18.74a	52.5a (46.50)	61.6c (51.87)
5.00	18.78a	47.5a (43.56)	41.5d (40.08)
10.00	13.07b	65.0a (57.76)	23.4e (28.45)
SEM ±	1.12	7.72	3.08

[a] Values are averages of four replicates with 10 eggs per replicate. Percentages were subjected to angular transformations and the values are given in parentheses.

[b] Calculated from number pupariating. In each column, values not followed by the same letter are significantly different ($P < 0.05$).

TABLE 2. DEVELOPMENT OF THIRD-INSTAR *Musca domestica* LARVAE REARED ON 3-EPICARYOPTIN-TREATED DIET[a]

Conc. (ppm)	Percent change in puparial weight (mg)[b]	Pupariation (%)	Emergence (%)[c]
0.0	+21.33a (27.47)	100.0a (90.0)	96.7a (83.5)
3.0	+06.94b (14.49)	100.0a (90.0)	86.7a (72.29)
6.0	+03.25b (09.60)	100.0a (90.0)	63.3b (52.86)
10.0	+02.66b (09.17)	96.7a (83.85)	58.1bc (50.07)
20.0	−28.43c (31.94)	93.33a (81.15)	40.0c (38.85)
40.0	−18.52c (25.45)	86.7b (68.85)	19.4d (25.86)
SEM ±	2.60	3.02	3.82

[a] Values are averages of three replications with 10 larvae per replicate. Percentages were subjected to angular transformation and the values are given in parentheses.
[b] Calculated as a percentage of the difference between puparial weight and that of third-instar larvae used.
[c] Calculated from number pupariating. In each column, values not followed by the same letter are significantly different ($P < 0.05$).

instar larvae, weights of larvae were determined at regular intervals and the number pupariating observed every 4 hr (Figures 1 and 2). The treatments used were those expected to give around 50% and 25% adult emergence from puparia as read from probit lines fitted by eye to data from Tables 1 and 2. With increasing concentrations of 3-epicaryoptin, there was a decrease in weight gain of the larvae. The first-instar larvae in the treated media continued to feed and increase in weight up to 102 hr, while the control larvae had stopped feeding at 96 hr (Figure 1). With the third-instar larvae, however, the larvae in the treatments stopped feeding and began to lose weight earlier than the controls (Figure 2). There was a significant difference between the time taken for 50% pupariation of control, treated, and starved larvae (Figures 1 and 2).

Although there was no significant difference in the rates of housefly pupariation (Tables 1 and 2), adult emergence was reduced in a dose-dependent manner. When the puparia were dissected two days after the last individuals had emerged, a few had fully developed melanized adults within. In the remaining

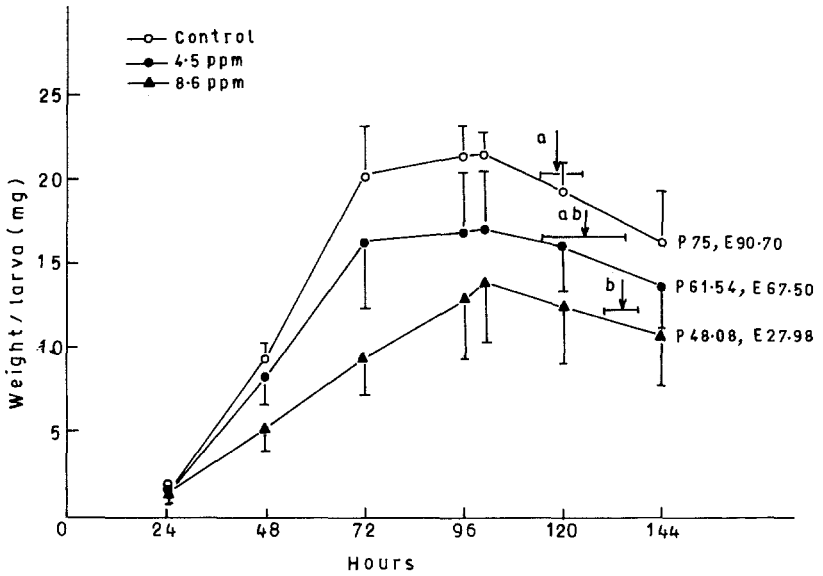


FIG. 1. Change in weight of first-instar *Musca domestica* larvae fed various concentrations of 3-epicaryoptin. Arrows indicate time of 50% pupariation. Arrows not followed by the same letter are significantly different ($P < 0.05$). Vertical and horizontal bars denote standard error. P and E represent percent pupariation and emergence, respectively. Each point in the graph represents an average of four replicates.

puparia, a mass of undifferentiated tissue contracted from the puparial wall was seen, indicating that death had occurred soon after pupariation. Larvae weighing around 14 mg when starved resulted in 100% pupariation and 95% emergence, but larvae of slightly lower weight (about 11.5 mg) when starved resulted in 70% pupariation and only 50% emergence (Figure 2).

The test compound was inactive in the molting hormone assay even at 1 μg/individual.

Exposure of fourth-instar *C. quinquefasciatus* larvae to graded treatments of 3-epicaryoptin (Table 3) resulted in mortality at different stages of development. At each treatment, mortality of larvae and pharate adults (PA_2) together accounted for more than 70% of the total kill. Death at the larva–pupal molt (L-P) was significant, and a few pupae died immediately after the molt at the white pupa stage (WP) at 2 ppm. Death at the PA_2 stage occurred at different times, starting from fully tanned and straight pupae to adults that had half emerged and were unable to extricate themselves fully from the pupal exuviae.

Staged fourth-instar mosquito larvae exposed to 2 ppm of the test compound for 2 hr exhibited sensitivity, while the WP were not affected (Table 4).

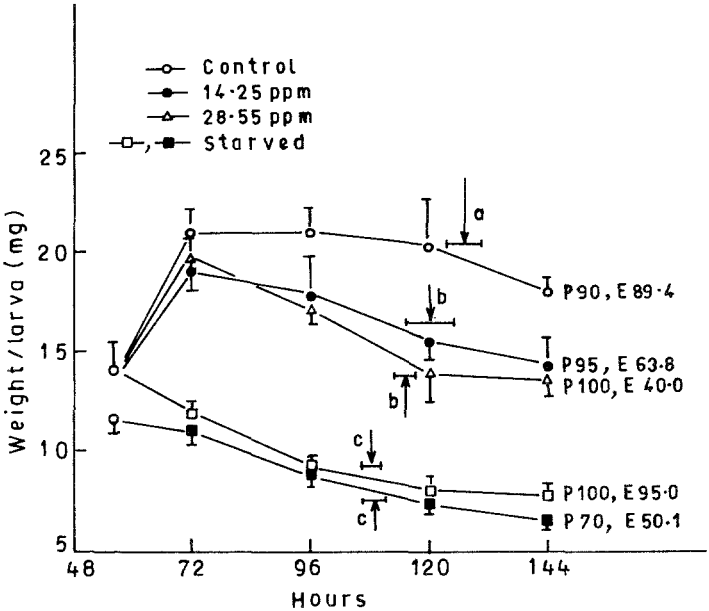


FIG. 2. Change in weight of third-instar *Musca domestica* larvae fed various concentrations of 3-epicaryoptin. Arrows indicate time of 50% pupariation. Arrows not followed by the same letter are significantly different ($P < 0.05$). Vertical and horizontal bars denote standard error. P and E represent percent pupariation and emergence, respectively. Each point in the graph represents an average of four replicates.

Although there was no significant difference in mortality when mid-fourth and late-fourth-instar larvae were exposed to the treatment, the pattern of kill was different. With the mid-fourth-instar larvae, mortality occurred at all the intervening stages of development, and the number dying at the WP stage accounted for about 40% of the total kill. With the late-fourth-instar larvae and pharate pupal (PP) stages, death at PA_2 stage accounted for about 90% of the total kill. Thus, apparently, the treatment effect was delayed by increasing the age of the larvae.

DISCUSSION

Hosozawa et al. (1974a, b) have demonstrated the antifeedant effect of 3-epicaryoptin against *Spodoptera litura* F. larvae. In the present study, with increasing concentrations of 3-epicaryoptin, there was a decrease in weight gain of the larvae, indicating an antifeedant effect (Figures 1 and 2). The reduced

TABLE 3. EFFECT OF 3-EPICARYOPTIN ON DEVELOPMENT OF FOURTH-INSTAR *Culex quinquefasciatus* LARVAE[a]

Conc. (ppm)	Percent[b] dead at stage					Emergence (%)[b]
	L	L–P	WP	P	PA_2	
0.0	11.00a (16.64)	0.0a (00.00)	0.0a (00.00)	1.92a (04.03)	0.0a (00.00)	87.08a (69.33)
0.25	16.00ab (24.50)	1.0ab (02.88)	0.0a (00.00)	3.0a (06.99)	8.0b (15.77)	70.00ab (57.27)
0.50	18.00ab (20.18)	3.0bc (13.98)	1.0a (02.89)	8.92b (16.99)	14.84bc (19.48)	56.28b (48.73)
1.00	32.0bc (34.32)	12.0c (17.60)	0.0a (00.00)	7.0b (16.94)	29.0c (32.39)	20.0c (25.18)
2.00	46.0c (42.25)	11.0c (18.70)	6.0b (09.52)	4.0ab (09.88)	30.0c (32.80)	3.0d (06.99)
SEM ±	5.04	4.08	3.24	2.58	4.66	4.30

[a] Values are averages of four replications with 25 larvae per replicate.
[b] Percentages were subjected to angular transformation and the values are given in parentheses. Values in each column not followed by the same letter are significantly different ($P < 0.05$).

TABLE 4. MORTALITY OF STAGED FOURTH-INSTAR *Culex quinquefasciatus* LARVAE EXPOSED TO 2 ppm OF 3-EPICARYOPTIN FOR 2 hr[a]

Stage exposed	Conc. (ppm)	Percent[b] dead at stage						Emergence (%)[b]
		L	PP	L–P	WP	P	PA_2	
Mid-fourth instar	2.0	5.0a (08.78)	5.0a (12.76)	2.0a (05.77)	12.25a (18.30)	1.0a (02.88)	6.0a (12.06)	71.00a (57.75)
Late fourth instar	2.0	0.0a (00.00)	2.08ab (05.91)	0.0a (00.00)	1.0b (02.88)	0.0a (00.00)	26.04b (30.52)	72.12a (58.39)
PP	2.0		0.0b (00.00)	1.0a (02.88)	3.0b (05.07)	0.0a (00.00)	40.37b (39.40)	55.66b (48.24)
WP	2.0				0.0b (00.00)	3.04a (08.73)	4.04a (09.95)	92.92c (74.72)
SEM ±		6.90	2.68	3.04	3.24	2.34	4.02	2.84
Mid-fourth instar	0.0	2.0	0.00[c]	0.00	0.00[c]	5.00[c]	2.00	91.00[c]
Late fourth instar	0.0	0.00	0.00	0.00	0.96	2.04	5.09[c]	91.91[c]
PP	0.0		0.00	0.00	1.00	2.04	4.04[c]	93.96[c]
WP	0.0				0.00	5.00	5.12	89.88

[a] Values are averages of four replicates with 25 larvae/replicate, except for WP having 15/replicate.
[b] Percentages were subjected to angular transformations and the values are given in parentheses. In each column, values not followed by the same letter are significantly different ($P < 0.05$).
[c] Control values are significantly different from those of their respective treatments on application of t test.

weight of puparia obtained from rearing third-instar larvae on treated diet (Table 2) was due to a combined effect of reduced feeding and early and steeper loss in weight prior to pupariation compared to the control (Figure 2). The early cessation of feeding and steeper loss in weight might partially account for the early pupariation of third-instar larvae. Azadirachtin, isolated from neem seed, when injected into mature third-instar blowfly larvae, also caused an early and steeper loss in weight prior to pupariation. There was, however, a slight delay in pupariation (Bidmon et al., 1986). With the first-instar larvae (Figure 1), the longer feeding period might be an attempt to attain a critical weight for pupariation. Thus, control larvae weighing around 14 mg, which is the highest weight attained by first-instar larvae in 8.6 ppm, when starved, resulted in 100% pupariated and 95% emergence. In larvae of a lower weight (around 11.5 mg) when starved only 70% pupariated and only 50% emerged (Figure 2).

Antifeedants also can induce growth-regulating effects (Rembold, 1984). With 3-epicaryoptin, adult emergence of flies was reduced in a dose-dependent manner even though the weights of puparia obtained from the treatments did not differ significantly from those of the control (Table 1). This indicated that growth inhibition by 3-epicaryoptin was not due to reduced feeding alone. This observation was supported by the normal emergence of starved larvae weighing 14 mg, whereas larvae of the same weight fed on 3-epicaryoptin resulted in a significantly lower emergence (Figure 2). Furthermore, staged fourth-instar mosquito larvae exposed to 2 ppm of 3-epicaryoptin for only 2 hr, showed reduced emergence (Table 4). The reduced adult emergence from larvae fed on 3-epicaryoptin thus could be due to a disturbance in the hormonal mileu of the developing insect.

20-Hydroxyecdysone has been reported to cause early pupariation in flies (Singh and Russell, 1980) and death at L-P moult in mosquitoes (Busvine et al., 1976). However, the lack of activity in the molting hormone (MH) assay excludes a MH-like mode of action of 3-epicaryoptin.

Dissection of unemerged puparia showing within a mass of undifferentiated contracted tissue or fully melanized adults and death at L-P molt/PA_2 stages in *C. quinquefasciatus* could relate to inhibition of the tanning or sclerotization process. It is interesting to note that azadirachtin, a tetranortriterpenoid isolated from neem seed, when fed to housefly and blowfly larvae caused a reduction in the puparial weight and eclosion rate (Wilps, 1986). Growth inhibition by azadirachtin was brought about by modifying the JH and ecdysteroid titers in the insect (Rembold, 1984). The mode of action of 3-epicaryoptin is not known and further work is in progress.

This is the first report of growth inhibition by the clerodane compound 3-epicaryoptin. A number of clerodane compounds have been isolated from various *Clerodendron* spp. (Hosozawa et al., 1974a, b) and synthesised (Belles et al., 1985; Kojima and Kato, 1981). None has been tested against dipteran insect pests, and this could be a fruitful line of research.

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3Z,6Z,9Z-TRIENES AND UNSATURATED EPOXIDES AS SEX ATTRACTANTS FOR GEOMETRID MOTHS[1]

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Abstract—Sex attractants for three species of geometrid moths were discovered during field screening of 3Z,6Z,9Z-trienes, and the racemic monoepoxydienes derived therefrom. Male *Probole amicaria* moths were attracted to lure blends containing 6Z,9Z-*cis*-3,4-epoxy-nonadecadiene (6Z,9Z-*cis*-3,4-epoxy-19:H) with 3Z,9Z-*cis*-6,7-epoxy-19:H. 3Z,6Z,9Z-19:H was positively identified and 6Z,9Z-*cis*-3,4-epoxy-19:H was tentatively identified in extracts of female pheromone glands by coupled gas chromatography–electroantennogram detection (GC-EAD) and gas chromatography–mass spectrometry (GC-MS). *Sicya macularia* male moths were attracted by blends of 3Z,6Z,9Z-19:H and 6Z,9Z-*cis*-3,4-epoxy-19:H. The attractive response was strongly antagonized by small amounts of 3Z,9Z-*cis*-6,7-epoxy-19:H, or by larger amounts of 3Z,6Z-*cis*-9,10-epoxy-19:H. *Lomographa semiclarata* male moths were attracted by a variety of lures containing 3Z,6Z,9Z-17:H as a major component. 3Z,6Z,9Z-17:H was tentatively identified in a female pheromone gland extract by GC-EAD.

Key Words—Lepidoptera, Geometridae, sex attractant, behavioral antagonist, 6Z,9Z-*cis*-3,4-epoxy-nonadecadiene, 3Z,9Z-*cis*-6,7-epoxy-nonadecadiene, 3Z,6Z,9Z-heptadecatriene, 3Z,6Z,9Z-nonadecatriene, *Probole amicaria*, *Sicya macularia*, *Lomographa semiclarata*.

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INTRODUCTION

The chemical elucidation of 3*Z*,6*Z*-*cis*-9,10-epoxy-heneicosadiene (3*Z*,6*Z*-*cis*-9,10-epoxy-21:H) as a component of the sex pheromone of the saltmarsh caterpillar moth, *Estigmene acrea* (Drury) (Hill and Roelofs, 1981) was the first in an ever-expanding series of structurally related sex pheromone components and sex attractants for male moths. Since this report, several monounsaturated and diunsaturated monoepoxide analogs of C_{17}–C_{21} hydrocarbons having unsaturation at the 3, 6, or 9 positions have been reported as male sex attractants for several species within the families Arctiidae (Hill et al., 1982; Descoins and Frérot, 1984; Bell and Meinwald, 1986), Noctuidae (Wong et al., 1985), and Geometridae (Becker et al., 1985, 1987; Wong et al., 1985; Millar et al., 1987).

As part of our continuing studies in this area, we have been investigating the sex attractant activity of 3*Z*,6*Z*,9*Z*-triene hydrocarbons in combination with both the racemic and chiral forms of diunsaturated epoxides of this type. We report here the elucidation of sex attractant blends for three geometrid moths, *Probole amicaria* Herrich-Schäffer, *Sicya macularia* Harris, and *Lomographa semiclarata* Walker.

In the work reported below, the following distinctions are made: an attractant is a compound with demonstrated attractant activity in field tests but which has not yet been identified in extracts of female moth abdominal tip extracts, while a compound designated as a pheromone component has demonstrated field activity, electroantennogram activity, and has been identified in insect extracts.

METHODS AND MATERIALS

Insects, Electroantennography, and Pheromone Identification. Female moths of unknown mating status were collected from the field using sweep nets or by black-light trapping and used as a source of pheromone. Male moths, obtained similarly or by capture in traps baited with synthetic sex attractants, were used for electroantennographic (EAG) and coupled gas chromatography–electroantennogram detection (GC-EAD) studies. Male antennal responses to synthetic compounds were measured by EAG (Chisholm et al., 1975) using 1-μg amounts of compounds applied to filter paper disks, and by the GC-EAD method of Arn et al. (1975). In GC-EAD studies, antennal and flame ionization detector responses were simultaneously recorded with a matched pair of Hewlett-Packard 3392A integrating chart recorders, which marked the retention times of eluting peaks directly on the chromatograms. Synthetic compounds tested included C_{17-22} 6*Z*,9*Z*- and 3*Z*,6*Z*,9*Z*-unsaturated hydrocarbons and their monoepoxydiene analogs.

Female sex pheromone gland extract was obtained by a 20-min pentane extraction of the terminal three to four abdominal segments of female moths. The extract was concentrated under a stream of nitrogen; internal standards [*n*-heptadecane (17:H) and *n*-tetracosane (24:H) were added, and the extract was submitted to GC-EAD as previously described (Millar et al., 1987). In all cases, a pheromone gland extract was analyzed first, followed by a standards run, thus eliminating the possibility of false results caused by contamination of the pheromone extract.

Insect Trapping. Field experiments were carried out in a mixed forest area approximately 100 km northeast of Saskatoon. Pherotech (Vancouver, British Columbia) wing-traps, similar in design to Pherocon 1CP traps (Zoecon Corp., Palo Alto, California) were used throughout and were baited with red rubber septa (Thomas Scientific, San Francisco, California) containing the synthetic lure blends. In addition to the replicated trap lures described in the tables, replicated survey traps ($N = 2$) were fielded continuously in 1984–1986 from the end of April to the beginning of October and were rebaited twice during each season. Survey traps were baited (500 μg total dose) with each of the C_{17-21} epoxydiene regioisomers as single components and in blends with their corresponding 3*Z*,6*Z*,9*Z* hydrocarbon. Traps were hung from branches 10–15 m apart at a height of ca. 1.5 m and were set out in randomized block designs. Moth captures were recorded twice per week and summed trap captures were transformed $[(X + 1)^{1/2}]$ and subjected to analysis of variance. The homogeneity of the variances of the transformed means was confirmed with Bartlett's test (Sokal and Rohlf, 1981). Treatments with zero trap captures overall were not included in the ANOVA, as replicates within a treatment must have some variance to satisfy the assumptions of the ANOVA test. Significantly different means were separated by Duncan's (1955) multiple range test. In tables, values within a column followed by a common letter are not significantly different ($P > 0.05$).

Synthetic Chemicals. Compounds used in the work described here (3*Z*,6*Z*,9*Z*-unsaturated hydrocarbons and the corresponding *cis*-3,4-, *cis*-6,7-, and *cis*-9,10-monoepoxydiene analogs) had been synthesized in this laboratory as previously described (Underhill et al., 1983; Wong et al., 1985; Millar et al., 1985, 1987). Chemical and isomeric purities for all compounds used were >98% by capillary GC (DB-1701).

RESULTS

Adult *Probole amicaria* males were captured in several traps baited with 6*Z*,9*Z*-*cis*-3,4-epoxy-19:H during 1984 field screening tests. This result was confirmed and extended in a replicated experiment testing the attractiveness of blends of 6*Z*,9*Z*-*cis*-3,4-epoxy-19:H with 3*Z*,6*Z*,9*Z*-19:H, 3*Z*,9*Z*-*cis*-6,7-

epoxy-19:H, or 3Z,6Z-*cis*-9,10-epoxy-19:H (Table 1). All lures containing the 3,4-epoxide were attractive. The blends of 6Z,9Z-*cis*-3,4-epoxy-19:H with 3Z,9Z-*cis*-6,7-epoxy-19:H (1 and 10% of the bait mixture, respectively) resulted in significantly higher trap catches than 6Z,9Z-*cis*-3,4-epoxy-19:H alone, indicating some synergism between the two components. None of the additives when tested alone were attractive.

This was the first time we had observed increased captures of male moths of any species as a result of testing bait mixtures composed of different positional epoxide isomers, and we were not aware of others reporting similar findings. As a result, a concerted effort was made to capture *P. amicaria* females from the field and ascertain if the field trapping data were a reasonably accurate reflection of this species' sex pheromone components. Coupled (GC-EAD) analysis of an abdominal tip extract of a single female moth of unknown mating status, using the antenna of a conspecific male, provided the support anticipated (Figure 1). Two male antennal stimulatory responses were elicited by the extract (Figure 1, lower traces), and these responses corresponded exactly in retention time with synthetic 3Z,9Z-*cis*-6,7-epoxy-19:H and 6Z,9Z-*cis*-3,4-epoxy-19:H (19B, Figure 1; order of epoxide elution was 6, 7, < 3, 4 < 9, 10). The antennal responses to synthetic C_{18-22} 3Z,6Z,9Z-triunsaturated hydrocarbons and their corresponding 6,7-, 3,4-, and 9,10-monoepoxides (Figure 1, upper pair of traces) were in complete accord with the field and the insect extract GC-EAD data, that is, the C_{19} 6,7- and 3,4-monoepoxides resulted in the strongest responses from the male antenna. A small peak with the same retention time as

TABLE 1. CAPTURE OF *Probole amicaria* MALES BY TRAPS BAITED WITH 3Z,6Z,9Z-19:H AND ITS MONEPOXIDE ANALOGS

Lure composition (μg)	Total males captured[a]
6Z,9Z-*cis*-3,4-epoxy-19:H (500)	38cde
6Z,9Z-*cis*-3,4-epoxy-19:H (500) + 3Z,6Z,9Z-19:H (5)	75bcd
6Z,9Z-*cis*-3,4-epoxy-19:H (500) + 3Z,6Z,9Z-19:H (50)	108abc
6Z,9Z-*cis*-3,4-epoxy-19:H (500) + 3Z,9Z-*cis*-6,7-epoxy-19:H (5)	191a
6Z,9Z-*cis*-3,4-epoxy-19:H (500) + 3Z,9Z-*cis*-6,7-epoxy-19:H (50)	116ab
6Z,9Z-*cis*-3,4-epoxy-19:H (500) + 3Z,6Z-*cis*-9,10-epoxy-19:H (5)	16e
6Z,9Z-*cis*-3,4-epoxy-19:H (500) + 3Z,6Z-*cis*-9,10-epoxy-19:H (50)	26de
3Z,9Z-*cis*-6,7-epoxy-19:H (50)	0
3Z,6Z-*cis*-9,10-epoxy-19:H (50)	0
3Z,6Z,9Z-19:H (50)	0
Blank	0

[a]Each treatment was replicated three times; traps were set out June 9–July 14, 1984. Values followed by the same letter are not significantly different ($P > 0.05$).

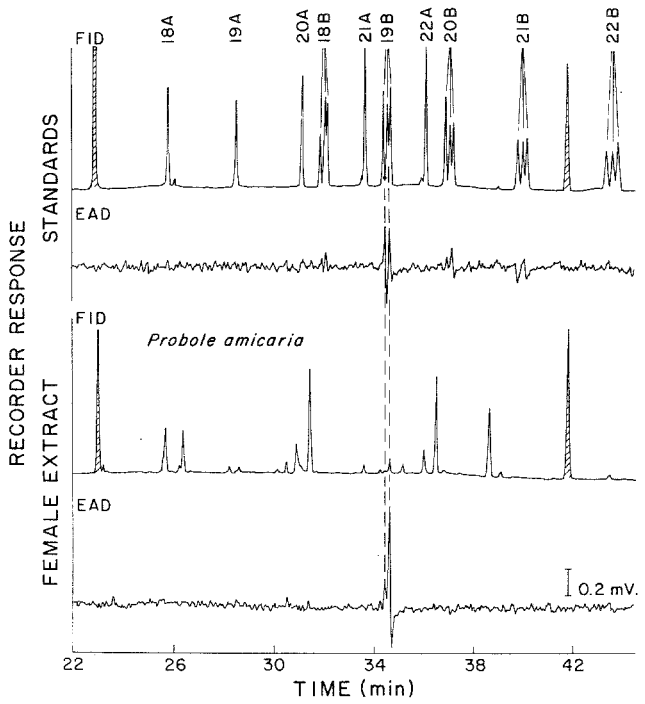


FIG. 1. Simultaneously recorded flame ionization detector (FID) and electroantennographic detector (EAD) traces using antennae of male *Probole amicaria*. Upper pair are in response to a mixture of C_{18-22} 3Z,6Z,9Z-triene hydrocarbons (designated by the letter A and preceded by the carbon chain length) and their monoepoxydiene hydrocarbon analogs (designated by the letter B); the latter eluted from the DB-1701 column in the order 6,7-, 3,4-, and 9,10-monoepoxydiene. Internal standards: 17:H and 24:H (shaded). Lower pair are in response to an abdominal tip extract of a conspecific female.

synthetic 3Z,6Z,9Z-19:H (Figure 1, 19A) also was present in the flame ionization detector chromatogram of the female insect extract.

A second abdominal tip extract from a field-collected female was analyzed by chemical ionization (isobutane) GCMS in selected ion monitoring (SIM) mode. A peak in the extract at the retention time corresponding to 3Z,6Z,9Z-19:H gave a good ion ratios match (five ions) for that compound [synthetic standard, 400 pg: *m/z* 319 (53%, M + 57), 263 (64%, M + 1), 261 (100%, M − 1), 221 (16%, M + 1 − 42), 206 (30%, M + 1 − 56); unknown in extract: *m/z* 319 (66%), 263 (54%), 261 (100%), 221 (14%), 206 (24%)], confirming the presence of 3Z,6Z,9Z-19:H in the pheromone gland extract. An excellent retention time match and a fair ion ratios match (two ions) also was obtained for a peak at the retention time corresponding to 6Z,9Z-*cis*-3,4-epoxy-

19:H [400 pg synthetic standard: *m/z* 279 (100%, M + 1), 261 (69%, M + 1 − 18); unknown in extract: *m/z* 279 (100%), 261 (59%)], suggesting the presence of 6*Z*,9*Z*-*cis*-3,4-epoxy-19:H in the pheromone gland extract. No peak was detected at the retention time of 3*Z*,9*Z*-*cis*-6,7-epoxy-19:H. Thus, the mass spectral data, in conjunction with the GC-EAD and field trapping data, suggest that 6*Z*,9*Z*-*cis*-3,4-epoxy-19:H is indeed a pheromone component. The presence of 3*Z*,6*Z*,9*Z*-19:H in the extract is unequivocal, but its role at this point is unclear. It is reasonable to suppose that it is a precursor to the attractive compound 6*Z*,9*Z*-*cis*-3,4-epoxy-19:H, but at low doses (1–9% of the total bait loading) it did not exhibit significant synergism (Table 1). It also did not give a significant antennal response in GC-EAD studies (Figure 1).

Antennae of *Sicya macularia* males have been found to be strongly stimulated in EAG studies (Wong et al., 1985) by combined monoepoxide mixtures (CME-19:H) of diunsaturated monoepoxides derived from 3*Z*,6*Z*,9*Z*-19:H, but traps baited with CME-19:H, alone or in combination with 3*Z*,6*Z*,9*Z*-19:H, failed to capture males of this species. Following chromatographic separation of the mixture of epoxides, it was found that 6*Z*,9*Z*-*cis*-3,4-epoxy-19:H was the most stimulatory positional isomer, eliciting EAG responses of ca. 5–6 mV.

During field trials in 1984, no male *S. macularia* were taken in survey traps baited with the three racemic epoxides as single components, but mixtures containing 6*Z*,9*Z*-*cis*-3,4-epoxy-19:H and 3*Z*,6*Z*,9*Z*-19:H (10:1) took 26 males. In a replicated test, traps baited with 9:1 and 4:1 mixtures of the *cis*-3,4-epoxide and triene captured maximally (Table 2). Furthermore, addition of

TABLE 2. CAPTURE OF *Sicya macularia* MALES IN TRAPS BAITED WITH MIXTURES CONTAINING 6*Z*,9*Z*-*cis*-3,4-epoxy-19:H AS THE MAJOR COMPONENT

Lure composition (μg)	Total males captured[a]
6*Z*,9*Z*-*cis*-3,4-epoxy-19:H (500)	0
6*Z*,9*Z*-*cis*-3,4-epoxy-19:H (500) + 3*Z*,6*Z*,9*Z*-19:H (5)	0
6*Z*,9*Z*-*cis*-3,4-epoxy-19:H (450) + 3*Z*,6*Z*,9*Z*-19:H (50)	36a
6*Z*,9*Z*-*cis*-3,4-epoxy-19:H (400) + 3*Z*,6*Z*,9*Z*-19:H (100)	23ab
6*Z*,9*Z*-*cis*-3,4-epoxy-19:H (250) + 3*Z*,6*Z*,9*Z*-19:H (250)	8c
6*Z*,9*Z*-*cis*-3,4-epoxy-19:H (400) + 3*Z*,6*Z*,9*Z*-19:H (100) + 3*Z*,9*Z*-*cis*-6,7-epoxy-19:H (5)	0
6*Z*,9*Z*-*cis*-3,4-epoxy-19:H (400) + 3*Z*,6*Z*,9*Z*-19:H (100) + 3*Z*,9*Z*-*cis*-6,7-epoxy-19:H (50)	0
6*Z*,9*Z*-*cis*-3,4-epoxy-19:H (400) + 3*Z*,6*Z*,9*Z*-19:H (100) + 3*Z*,6*Z*-*cis*-9,10-epoxy-19:H (5)	14bc
6*Z*,9*Z*-*cis*-3,4-epoxy-19:H (400) + 3*Z*,6*Z*,9*Z*-19:H (100) + 3*Z*,6*Z*-*cis*-9,10-epoxy-19:H (50)	5c

[a]Each treatment was replicated ($N = 3$) July 25–August 29, 1984. Values followed by the same letter are not significantly different ($P > 0.05$).

the racemic 6,7-epoxide isomer at the 1% level of total lure dose completely suppressed male captures; additions of the 9,10-epoxide isomer at the 10% level decreased captures but did not cause total suppression of the attraction.

Approximately 190 males of another geometrid species, *Lomographa semiclarata* (Ennominae, Baptini), were captured in traps baited with 3Z,6Z,9Z-17:H as a lure component common to all traps. In GC-EAD studies with synthetic standards, mixtures containing 3Z,6Z,9Z-trienes, and the combined monoepoxides derived therefrom, male antennae were most strongly stimulated by the C_{17} triene (Figure 2, upper traces), with diminishing responses to the C_{18} to C_{21} homologs. An abdominal tip extract was obtained from a field-collected female of unknown mating status. GC-EAD analysis revealed a single, major, antennal-stimulatory component in the extract that corresponded exactly in retention time to synthetic 3Z,6Z,9Z-17:H (Figure 2, lower traces). Thus, all data suggest this species utilizes only a single component pheromone.

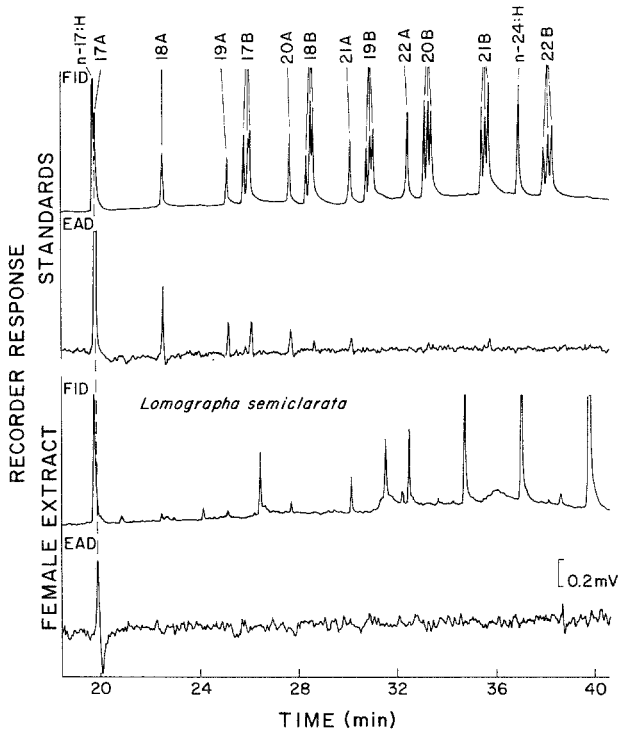


FIG. 2. FID and EAD traces (antennae of male *Lomographa semiclarata*); upper pair are in response to synthetic C_{17-22} triene hydrocarbons (A), and monoepoxydiene hydrocarbon analogs (B); number/letter designation of compounds is similar to that given in Figure 1; lower pair of traces are in response to an abdominal tip extract (1 FE) from a conspecific female. A DB-1701 column was used. Internal standards: 17:H and 24:H.

DISCUSSION

The seminal paper in this series (Wong et al., 1985) reported preliminary results of field tests with unsaturated triene hydrocarbons and epoxides derived therefrom; results were reported for 26 species in the geometrid and noctuid families. This, plus reports from other groups (Bell and Meinwald, 1985, and references therein; compilation to 1986, Arn et al., 1986) indicated very clearly how widespread these types of pheromones may be and that there were multiple synergistic and antagonistic interactions at work. We felt that these multiple interactions could only be worked out by having the entire range of structural possibilities to work with.

A large synthetic program was undertaken, and we have available now the entire range of trienes plus epoxides derived therefrom, in chain lengths C_{16-22}. The results from this work have been considerable, both in terms of the number of species caught (approximately 80 in our area of central Canada alone), and in terms of unraveling the complex interplay of synergists and inhibitors, which the preliminary work by Wong et al. (1985) had indicated.

Our approach has been to set out a large number of synthetic baits, both as individual compounds and as blends. This has given us a large amount of field-trapping data and has provided male moths for EAG and GC-EAD studies. However, it has not provided female insects, and, in fact, for a number of species, we have not been able to obtain females for the analysis of pheromone glands, despite extensive sweep-netting and light-trapping.

There were indications in the study by Wong et al. (1985) that multiple chiral and regioisomeric components could be involved in lepidopteran attractants. To our knowledge, the attractant blend for *P. amicaria* represents the first example in the Lepidoptera of a blend involving two potentially chiral components and the first example of an attractant blend of two regioisomers. Only one of the two components (6*Z*,9*Z*-*cis*-3,4-epoxy-19:H) has any activity alone, and it is strongly synergized by the second component. The 9,10-epoxide regioisomer is apparently benign, causing no apparent synergism or antagonism.

The presumed precursor of the attractive epoxides, 3*Z*,6*Z*,9*Z*-19:H, was present in female abdominal tip extracts but gave no significant increases in trap captures when incorporated into baits at low levels.

A second species, *S. macularia*, was attracted to traps baited with mixtures of 3*Z*,6*Z*,9*Z*-19:H and 6*Z*,9*Z*-*cis*-3,4-epoxy-19:H. These compounds were mutually synergistic, as neither was active alone. Trap captures were completely suppressed by addition of small quantities of 3*Z*,6*Z*-*cis*-6,7-epoxy-19:H. Consequently, *S. macularia* males were not cross attracted by the optimal sex attractant for *P. amicaria*, which contained an appreciable amount of 3*Z*,9*Z*-*cis*-6,7-epoxy-19:H.

The responses of *S. macularia* males were also significantly antagonized by somewhat larger amounts of 3Z,6Z-*cis*-9,10-epoxy-19:H, which is known to be a sex attractant for a number of sympatric species (Wong et al., 1985). Thus the responses of male *S. macularia* were characterized both by the specificity of the attractant blend and by sensitivity to several behavioral antagonists that are sex attractants for sympatric species.

The last species, *Lomographa semiclarata*, apparently produces 3Z,6Z,9Z-17:H, and is highly attracted to any bait containing 3Z,6Z,9Z-17:H, regardless of whatever other components may be there. This indiscriminate attraction would seem to be a very inefficient mate-location strategy, as there are a number of sympatric species that also use 3Z,6Z,9Z-17:H as part of their attractant blends (Millar et al., 1987). This suggests that temporal or spatial factors, such as a specific period for male flight activity, must limit the responses of males of this species, as it would be energetically very expensive to the male insects to be attracted over a long distance to every triene emitter, many of which would be of a different species.

It is noteworthy that *P. amicaria* and *S. macularia* were both attracted by the racemic forms of the epoxides. Among the sex attractants incorporating epoxides of this type, one of the two possible enantiomers is usually responsible for the attraction, and the opposite enantiomer may be a strong behavioral antagonist (Wong et al., 1985; Becker et al., 1987). The question of the chirality of the attractive components of the sex attractants reported here is addressed in the accompanying paper (Millar et al., 1990).

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SYNTHESIS AND FIELD TESTING OF ENANTIOMERS OF 6Z,9Z-*cis*-3,4-EPOXYDIENES AS SEX ATTRACTANTS FOR GEOMETRID MOTHS

Interactions of Enantiomers and Regioisomers[1]

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Abstract—Stereoselective syntheses of chiral C_{17} to C_{21} 6Z,9Z-*cis*-3,4-epoxydienes were developed. Field tests of the enantiomerically enriched epoxides as components of synthetic sex attractant lures were carried out, and those with C_{17} and C_{19} chain lengths, particularly, were attractive to male moths of several species. Moths were usually specifically attracted by one of a pair of enantiomers, and the opposite enantiomer could actually be a behavioral antagonist. Males belonging to nine species of Geometridae were captured. *Probole amicaria* (Herrich-Schäffer) males were taken in traps baited with the mixture (6Z,9Z,3S,4R)-epoxy-nonadecadiene (6Z,9Z,3S,4R-epoxy-19:H) + 3Z,9Z,6R,7S-epoxy-19:H + 3Z,6Z,9Z-19:H(9:1:8). Other species responding to the C_{19} compounds included (attractant components follow in parentheses); *Sicya macularia* (Harris) (6Z,9Z,3S,4R-epoxy-19:H + 3Z,6Z,9Z-19:H), *Anavitrinella pampinaria* (Guenée) (6Z,9Z-*cis*-3,4-epoxy-19:H + 3Z,9Z,6S,7R-epoxy-19:H), and *Lycia ursaria* (Walker) (6Z,9Z-3S,4R-epoxy-19:H + 3Z,6Z,9Z-19:H). Males of the following species were captured by C_{17} epoxides: *Itame occiduaria* (Packard) (6Z,9Z,3R,4S-epoxy-17:H + 3Z,6Z,9Z-17:H), *Itame brunneata* (Thunberg) [6Z,9Z,3S,4R-epoxy-17:H), *Epelis truncataria* (Walker) (both enantiomers of 6Z,9Z-*cis*-3,4-

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epoxy-17:H), *Semiothisa ulsterata* (Pearsall) (3*Z*,9*Z*-6*S*,7*R*-epoxy-17:H), and *S. signaria dispuncta* (Walker) (3*Z*,9*Z*-*cis*-6,7-epoxy-17:H + 3*Z*,6*Z*,9*Z*-17:H). The interactions among enantiomers and regioisomers are discussed as a mechanism by which cross attraction between sympatric species is limited.

Key Words—Lepidoptera, Geometridae, sex pheromone, sex attractant, behavioral antagonist, enantiomer, (6*Z*,9*Z*,3*S*,4*R*)-epoxy-heptadecadiene, (6*Z*,9*Z*,3*R*,4*S*)-epoxy-heptadecadiene, (3*Z*,9*Z*,6*S*,7*R*)-epoxyheptadecadiene, (6*Z*,9*Z*,3*S*,4*R*)-epoxy-nonadecadiene, (6*Z*,9*Z*,3*R*,4*S*)-epoxy-nonadecadiene.

INTRODUCTION

During the last decade there have been a number of reports of lepidopteran sex attractants or sex pheromones containing mono- or diunsaturated epoxides as components (Hill et al., 1982; Descoins and Frérot, 1984; Bell and Meinwald, 1986; Wong et al., 1985; Becker et al., 1985; Millar et al., 1987). *cis*-Disubstituted epoxide sex attractants of this type have two enantiomeric forms, and several studies have demonstrated that chirality can play an important part in determining the attractive behavioral response to a particular sex attractant blend (Wong et al., 1985; Becker et al., 1987; Millar et al., 1987). The attractive response usually appears to be due to one of the two enantiomers, with the opposite enantiomer having a broad spectrum of biological activity, varying from synergizing to antagonizing the behavioral response to a sex attractant blend.

As part of our continuing studies on sex attractants and sex pheromones of geometrid and noctuid moths, we have been investigating the sex attractant activity of both the racemic and chiral forms of mono- and diunsaturated epoxides. We report here: (1) the synthesis of enantiomerically enriched forms of both of the enantiomers of 6*Z*,9*Z*-*cis*-3,4-epoxydienes of chain lengths C_{17-21} (Millar et al., 1985), (2) the results of field trails of enantiomerically enriched forms of 6*Z*,9*Z*-*cis*-3,4-epoxy-17:H and 6*Z*,9*Z*-*cis*-3,4-epoxy-19:H, (3) the elucidation of sex attractant blends for four geometrid moth species incorporating one enantiomer of 6*Z*,9*Z*-*cis*-3,4-epoxy-19:H, (4) the elucidation of sex attractant blends for four geometrid moth species incorporating one or both enantiomers of 6*Z*,9*Z*-*cis*-3,4-epoxy-17:H, (5) the identification of 3*Z*,9*Z*,6*S*,7*R*-epoxy-17:H as a sex attractant for the geometrid species *Semiothisa ulsterata*, and (6) the elucidation of a sex attractant for *Semiothisa signaria dispuncta* as a blend of the enantiomers of 3*Z*,9*Z*-*cis*-6,7-epoxy-17:H with 3*Z*,6*Z*,9*Z*-17:H.

In the work reported below, the following distinctions are made: an attractant is a compound with demonstrated attractant activity in field tests but which has not yet been identified in extracts of female moth abdominal tip extracts; a

compound designated as a pheromone component has demonstrated field activity, electroantennogram activity, and has been identified in insect extracts.

METHODS AND MATERIALS

Insects, Electroantennography, and Pheromone Identification

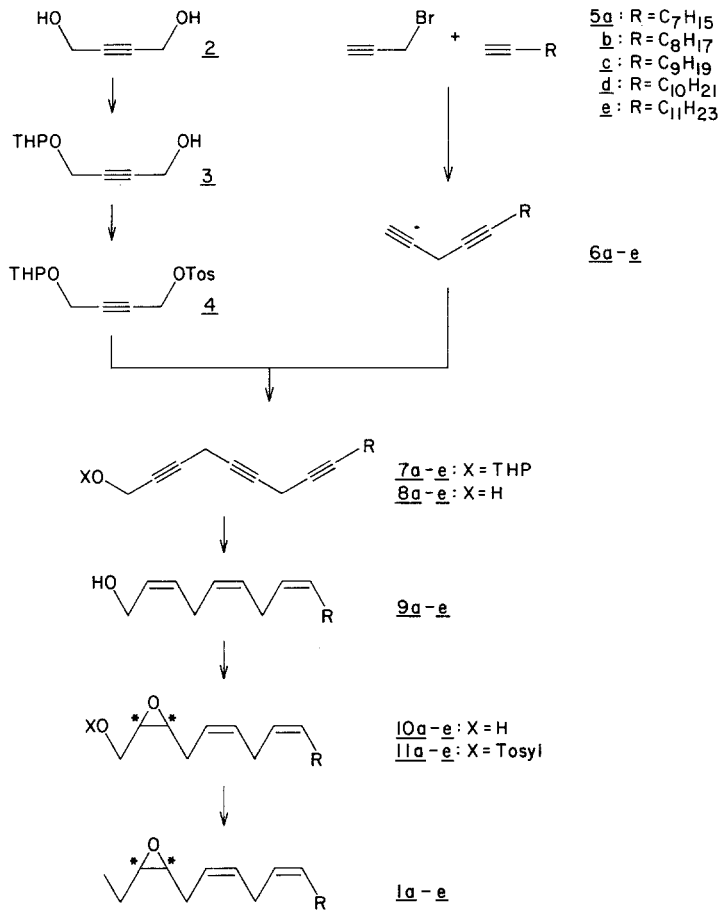
Methods used in collection of male and female insects, screening of synthetic compounds and pheromone gland extracts by electroantennography (EAG) and coupled gas chromatography–electroantennogram detection (GC-EAD), and the analysis of pheromone gland contents by coupled gas chromatography–mass spectrometry (GC-MS) were as described in the companion paper (Millar et al, 1990). The synthesis of all compounds, other than the enantiomerically enriched C_{17-21} 6*Z*,9*Z*-*cis*-3,4-epoxydienes, and the field testing protocol are also described in detail in the companion paper.

Synthesis of Enantiomerically Enriched Forms of C_{17-21} 6Z,9Z-cis-3,4-Epoxydienes

All reactions were carried out in oven-dried glassware. THF was distilled from sodium benzophenone ketyl under N_2 before use. Proton NMR spectra were recorded at 360 MHz on a Bruker AM-360-WB spectrometer. IR spectra were recorded on a Perkin-Elmer 237B instrument, as liquid films on NaCl plates or as CH_2Cl_2 solutions. EI and CI mass spectra were recorded on a Finnigan 4000E GC-MS unit with an Incos 2300 data system, using an SP-2100 capillary GC column. Optical rotations were measured with a Perkin-Elmer 141 polarimeter, using a 1-ml, 1-dm cell. Elemental analyses were performed by Spang Microanalytical Laboratory, Eagle Harbour, Michigan. Flash chromatography was performed with 230–400 mesh silica gel (EM Reagents). Where required, silver nitrate-impregnated silica gel was prepared by slurrying flash chromatography grade silica gel in acetonitrile solutions of $AgNO_3$ and evaporating off the solvent under vacuum.

Preparation of 2,5,8-Triynols 8. Dihydropyran (42 g, 500 mmol) was added dropwise over 6 hr to a solution of 2-butyn-1,4-diol **2** (43 g, 500 mmol) and *p*-toluenesulfonic acid (0.5 g, 2.6 mmol) in CH_3CN, and the solution was stirred overnight (Scheme 1). $NaHCO_3$ (2 g, 24 mmol) was added, and the mixture was stirred 30 min, then concentrated on a rotary evaporator. The residue was poured into water (150 ml) and extracted with ether (1 × 150 ml, 2 × 50 ml). The combined ether extracts were washed with water and brine (50 ml of each), dried (Na_2SO_4), concentrated, and fractionally distilled, giving 4-(tetrahydro-2-pyranyloxy)-2-butyn-1-ol **3** (34 g, 40%), bp 90–95°C (0.1 torr); lit bp 122–124°C (0.8 torr) (Arai and Ichikizawa, 1961).

The alcohol **3** was converted to the tosylate **4** exactly as described by Eiter et al. (1978). The crude tosylate was used directly without further purification.



SCHEME 1.

Diynes **6** were prepared exactly as described by Millar and Underhill (1986b), by CuI-catalyzed reaction of the anions of the alkynes **5** with propargyl bromide.

Diyne **6b** (7.39 g, 42 mmol) was added dropwise at 20°C to a freshly prepared solution of ethyl magnesium bromide (approx. 45 mmol) in THF (60 ml). The solution was warmed to 40°C for 1.5 hr, then cooled to 0°C, CuI (152 mg, 0.8 mmol) was added, and the mixture was stirred 15 min. The mixture was then cooled to ≤ 10°C, and crude tosylate **4** (13.6 g, approx. 42 mmol) in THF (20 ml) was added dropwise over 1 hr. The mixture was warmed to 20°C over 3 hr, and stirred overnight. The mixture was then poured into aq. NH_4Cl (1 M; 100 ml) and extracted with ether (3 × 100 ml). The combined

extracts were dried (Na_2SO_4) and concentrated. Toluene (50 ml) was added, and the solution was concentrated again to azeotrope traces of water, giving crude triyne **7b** as a brown oil.

The crude triyne **7b** was taken up in dry methanol (100 ml), *p*-toluenesulphonic acid (0.5 g, 2.6 mmol) was added, and the solution was stirred 2 hr at 20°C. $NaHCO_3$ (3 g, 36 mmol) was added, the solvent was removed, and the residue was poured into water (100 ml). The mixture was extracted with ether (1 × 100 ml, 2 × 50 ml), the combined ether extracts were washed with brine (1 × 50 ml), dried (Na_2SO_4), concentrated, and pumped under vacuum (0.5 torr). The semicrystalline residue was recrystallized from hot hexane, yielding trialkynol **8b** (7.1 g, 69% from **3**) as fine pale yellow plates mp 46.5°C. [^{1}H]NMR ($CDCl_3$): δ 4.23 (t, 2H, J = 2.1 Hz; H-1), 3.18 (tt, 2H, J = 2.1, 2.1 Hz; H-4), 3.11 (tt, 2H, J = 2.4, 2.1 Hz; H-7), 2.12 (tt, 2H, J = 7.1, 2.4 Hz; H-10), 1.71 (br s, 1H; OH), 1.46 (tt, 2H, J = 7.1, 7.1 Hz; H-11), 1.4–1.2 (m, 10H; H-12 to H-16), 0.85 (t, 3H, J = 6.8 Hz; H-17). IR (CH_2Cl_2): λ_{max} 3610 (s), 2930 (s), 2870 (s), 2260 (w), 2220 (w), 1010 (s) cm^{-1}. MS: 197 (trace; M-47), 183 (2.2), 169 (6.7), 155 (14.6), 141 (42.6), 131 (25.6), 129 (42.0), 128 (43.6), 117 (39.7), 115 (73.8), 105 (21.9), 103 (17.0), 95 (31.5), 91 (100), 81 (65.8), 79 (43.2), 77 (41.8), 67 (65.6), 65 (26.9), 57 (34.5), 55 (92.0), 53 (28.5), 51 (30.0).

The homologous triynols **8** were prepared in identical fashion in 75–85% yields, using the respective diynes **7.** Melting points: **8a** 44.5°C; **8c** 50–51°C; **8d** 55–56°C; **8e** 60°C. All compounds gave satisfactory NMR and IR spectra. The EI mass spectra were very similar to that of **8b**, except that the ion at *m/z* 55 increased in intensity with molecular weight, becoming the base peak for **8d** and **8e**. The triynols **8** also gave unusual CI (isobutane) mass spectra, with a moderate M + 1 ion (~20%) and a base peak at M + 57.

Preparation of 2Z,5Z,8Z-trienols **9**. (a) Catalytic reduction: 2,5,8-Heptatriyn-1-ol **8b** (6.76 g, 27.7 mmol) was dissolved in ethyl acetate (100 ml) and Lindlar catalyst (5% Pd on calcium carbonate, poisoned with Pb; 1.00 g) was added. The mixture was stirred for 10 min, then filtered with suction through a 1-cm pad of Celite. The filtrate was then added to a mixture of Lindlar catalyst (1.5 g) and pyridine (2 ml) in ethyl acetate (50 ml), and the mixture was flushed with argon for 5 min, then with H_2 for a further 5 min. The flask was sealed and stirred under a slight positive pressure of H_2. Reaction progress was monitored by GC. After 3 hr, the reduction was complete. The reaction mixture was filtered through Celite, the filtrate was concentrated, toluene (50 ml) was added, the solution was concentrated again (to azeotrope out traces of pyridine), and pumped on a vacuum line to remove the last traces of solvent. The crude product was purified by flash chromatography (silica gel; 30% ether in hexane), yielding trienol **9b** (5.50 g, 79%). [^{1}H]NMR ($CDCl_3$): δ 5.62 (dtt, 1H, J = 10.8, 6.6, 1.3 Hz; H-2), 5.52 (dtt, 1H, J = 10.8, 7.3, 1.3 Hz; H-3), 5.43–

5.26 (m, 4H; H-5, H-6, H-8, H-9), 4.21 (br dd, 2H, J = 6.6, ~6 Hz; H-1), 2.84 (t, 2H, J ~ 7 Hz; H-4), 2.78 (5, 2H, J ~ 7 Hz; H-7), 2.02 (m, 3H; H-10, OH), 1.4–1.1 (m, 12H; H-11 to H-16), 0.86 (t, 3H, J = 6.8 Hz; H-17). IR (neat): λ_{max} 3340 (br s), 3020 (m), 2960 (m), 2930 (s), 2880 (sh), 1020 (m) cm^{-1}. MS: 250 (trace, M^+), 232 (0.9), 219 (0.3), 206 (0.3), 191 (0.8), 178 (1.4), 166 (1.8), 147 (1.6), 138 (4.3), 133 (5.4), 119 (13.3), 110 (9.3), 109 (11.1), 105 (24.3), 95 (28.4), 93 (25.5), 91 (45.3), 81 (46.9), 80 (61.7), 79 (92.1), 69 (34.0), 67 (100), 57 (38.7), 55 (80.3).

The homologous trienols **9**, with the exception of **9d** (see below), were similarly prepared from the corresponding triynols **8** in 82–86% yield. The NMR and IR spectra were analogous to those of **9b**. The mass spectra were very similar to that of **9b** in the mass range *m/z* 45–150. The higher mass range was characterized by small but distinct ions at M-18, M-31, M-44, M-59, M-72, and M-84.

(b) Reduction of 2,5,8-Triyn-l-ol **8d** *by Hydroboration:* Several attempts to catalytically reduce triynol **8d** were not successful. Consequently the THP protecting group was reattached by treatment of **8d** (6.5 g, 24 mmol) in dry ether (100 ml) with dihydropyran (2.52 g, 30 mmol) for 48 hr at 20°C, with acid catalysis with a few crystals of PTSA. The reaction was worked up in the usual fashion (see above). The crude product was pumped under high vacuum for several hours to remove traces of dihydropyran, and then used directly in the next step.

Cyclohexene (14.9 g, 182 mmol) was added dropwise to a solution of borane–dimethylsulfide complex (10 M; 9.0 ml, 90 mmol) in THF (150 ml) at −5°C under Ar. The mixture was stirred for 30 min at −5°C, then warmed to 20°C, and stirred 1.5 hr. The mixture then was cooled to 0°C, and a solution of the crude protected triyne (8.47 g, 24 mmol) in THF (20 ml) was added dropwise over 30 min, maintaining the temperature at <5°C. The mixture was then warmed to 20°C and stirred 2.5 hr. Glacial acetic acid (40 ml) then was added, and the mixture was stirred overnight. The mixture was cooled to 0°C, aq. NaOH (5 M; 150 ml) was added dropwise, maintaining the temperature at <5°C, followed by aq. H_2O_2 (30%; 20 ml) (CAUTION, HIGHLY EXOTHERMIC!). The mixture was warmed to 20°C and extracted with hexane (1 × 200 ml, 2 × 100 ml). The combined hexane extracts were dried (Na_2SO_4), concentrated, and pumped under vacuum (0.1 torr) at 40°C for 2 hr to remove the considerable quantities of volatile by-products. The residue was taken up in methanol (100 ml) containing 0.5 g PTSA, and stirred overnight. Na_2CO_3 (2 g) then was added, and the methanol was removed on a rotary evaporator. The residue was poured into water (100 ml) and extracted with ether (3 × 75 ml). The combined ether extracts were washed with water, dried (Na_2SO_4), and concentrated, and the residue was purified by flash chromatography (silica gel, 30%

ether in hexane), yielding trienol **9d** (3.59 g, 54%). Spectra were in accord with those of the homologous compounds.

Asymmetric Epoxidation of (2Z,5Z,8Z)-trienols. To 50 ml of dry CH_2Cl_2 at −25°C under Ar was added sequentially titanium isopropoxide (3.27 ml, 11 mmol), (+)-diisopropyl tartrate (2.75 ml, 13.5 mmol), trienol **9b** (2.75 g, 11 mmol), and *t*-butyl hydroperoxide (4.75 M; 5.26 ml, 25 mmol), with vigorous stirring for 10 min between each addition. The resulting mixture was stirred for 30 min, and then sealed and stored at −30°C for nine days. The mixture was worked up by dropwise addition of aq. tartaric acid (10%, 27.5 ml), maintaining the temperature < −20°C, while stirring vigorously. The resulting slurry was stirred for a further 30 min at −25°C, warmed to 20°C over 2 hr, and stirred until the aq. layer was clear (~4 hr). The layers then were separated and the aqueous layer was extracted further with CH_2Cl_2 (2 × 10 ml). The combined CH_2Cl_2 layers were concentrated on a rotary evaporator. The residue was taken up in ether (50 ml), cooled to 0°C, and aq. NaOH (2M; 10 ml) was added. The mixture was stirred at 0°C until TLC indicated that all diisopropyl tartrate had been consumed (~45 min). The layers were then separated, and the aq. phase was extracted with ether (2 × 10 ml). The combined ether extracts were washed with brine, dried (Na_2SO_4), concentrated, and the residue was purified by flash chromatography (25% EtOAc in hexane), yielding 1.73 g (50%; 75% based on recovered starting material) of (2*S*,3*R*)-**10b**. [^{1}H]NMR ($CDCl_3$): δ 5.51 (dtt, 1H, *J* = 10.6, 7.3, 1.5 Hz; H-5), 5.45–5.35 (m, 2H, H-6 and H-8), 5.29 (dtt, 1H, *J* = 10.7, 7.1, 1.4 Hz; H-9), 3.85 (ddd, 1H, *J* = 11.8, 7.1, 4.5 Hz; H-1), 3.72 (ddd, 1H, *J* = 11.8, 6.6, 5.4 Hz; H-1′), 3.15 (ddd, 1H, *J* = 6.6, 4.5, 4.3 Hz; H-2), 3.06 (ddd, 1H, *J* = 6.6, 6.6, 4.3 Hz; H-3), 2.77 (br t, 2H, *J* = 7.1 Hz; H-7), 2.46 (m, 1H; H-4), 2.25 (m, 1H; H-4′), 2.02 (m, 2H; H-10), 1.66 (dd, 1H, *J* = 7.1, 5.4 Hz; OH), 1.24 (m, 12H; H-11 to H-16), 0.86 (t, 3H, *J* = 6.8 Hz; H-17). IR (neat): λ_{max} 3450 (br, m), 3020 (m), 2960 (s), 2930 (s), 2880 (sh), 2860 (s), 1045 (br, m) cm^{-1}. MS: 266 (trace; M^+), 248 (trace), 235 (1.6), 217 (1.5), 205 (1.1), 192 (1.9), 163 (0.8), 149 (1.7), 135 (5.5), 121 (8.0), 109 (13.1), 107 (13.4), 105 (11.1), 95 (26.4), 93 (50.9), 91 (37.4), 83 (22.2), 81 (45.5), 80 (71.5), 79 (100), 77 (24.7), 69 (29.1), 67 (79.5), 61 (17.6), 57 (42.3), 55 (72.1).

(2*R*,3*S*)-**10b** was prepared in a similar manner, using (−)-diisopropyl tartrate to form the chiral catalyst. All spectra (NMR, IR, and MS) matched those of (2*S*,3*R*)-**10b.**

The homologous compounds were prepared in identical fashion. NMR and IR spectra were analogous to those of **10b.** Mass spectra were characterized by ions of low intensity at M^+, M-18, M-31, M-49, M-61, M-74, and by a ion of moderate intensity at *m/z* 61. The base peak in all cases was at *m/z* 79.

The enantiomeric excess (ee) of each epoxyalcohol was determined by

esterification of the alcohol with (2*S*)-2-acetoxypropionyl chloride (Doolittle and Heath, 1984; Slessor et al., 1985), followed by GC (isothermal) on a DB-210 column (30 m × 0.25 mm). Baseline separation was achieved for compounds **10a–d.** Optical rotations, (2*S*,3*R*) series: **10a,** ee: 69%, $[\alpha]_D^{23} = -4.7°$ (c = 2.67, CH_2Cl_2); **10b,** ee: 58%, $[\alpha]_D^{23} = -2.9°$ (c = 2.29, CH_2Cl_2); **10c,** ee: 71%, $[\alpha]_D^{23} = -3.4°$ (c = 3.11, CH_2Cl_2); **10d,** ee: 90%, $[\alpha]_D^{23} = -3.4°$ (c = 2.73, CH_2Cl_2); **10e,** ee: 87%, $[\alpha]_D^{23} = -3.4°$ (c = 2.71, CH_2Cl_2). (2*R*,3*S*) series: **10a,** ee: 69%, $[\alpha]_D^{23} = +4.6°$ (c = 2.67, CH_2Cl_2); **10b**, ee: 57%, $[\alpha]_D^{23} = +3.3°$ (c = 1.81, CH_2Cl_2);**10c,** ee: 60%, $[\alpha]_D^{23} = +3.3°$ (c = 4.36, CH_2Cl_2); **10d,** ee: 81% $[\alpha]_D^{23} = +4.0°$ (c = 2.83, CH_2Cl_2); **10e**, ee: 81%, $[\alpha]_D^{23} = +3.8°$ (c = 2.52, CH_2Cl_2).

Preparation of Diene Tosylates **11a-e.** A solution of (2*S*,3*R*) epoxyalcohol **10b** (1.30 g, 4.89 mmol) and tosyl chloride (1.05 g, 5.5 mmol) in dry ether (50 ml) was cooled to $< -10°C$ in an ice-salt bath. Powdered KOH (2.0 g, 36 mmol) was added in four portions at 5-min intervals, with vigorous stirring. The resulting mixture was warmed to 0°C, and stirred a further 1.5 hr. The mixture was then poured into water (100 ml), the layers were separated, and the aqueous phase was extracted again with ether (2 × 25 ml). The combined ether extracts were washed with brine, dried (Na_2SO_4), concentrated, and pumped under vacuum (0.1 torr) to remove traces of solvent. The resulting yellow oil **11b** (2.07 g, quant.) gave one spot on TLC and was used without further purification. [^{1}H]NMR ($CDCl_3$): δ 7.80 (d, 2H, J = 8.4 Hz; aromatic H, ortho), 7.34 (d, 2H, J = 8.4 Hz; aromatic H, para), 5.49 (dtt, 1H, J = 10.7, 7.3, 1.5 Hz; H-6), 5.39 (dtt, 1H, J = 10.7, 7.3, 1.5 Hz; H-5), 5.34 (dtt, 1H, J = 10.7, 7.3, 1.5 Hz; H-9), 5.25 (dtt, 1H, J = 10.7, 7.3, 1.4 Hz; H-8), 4.20 (dd, 1H, J = 11.3, 5.0; H-1), 4.10 (dd, 1H, J = 11.3, 6.4 Hz; H-1′), 3.15 (ddd, 1H, J = 6.4, 5.0, 4.3 Hz; H-2), 2.99 (ddd, 1H, J = 6.4, 6.4, 4.3 Hz; H-3), 2.72 (dd, 2H, J = 7.2, 7.2 Hz; H-7), 2.43 (s, 3H; Me-Ar), 2.31 (m, 1H; H-4), 2.14 (m, 1H; H-4′), 2.01 (m, 2H; H-10), 1.4–1.2 (m, 12H; H-11 to H-16), 0.86 (t, 3H, J = 6.8 Hz; H-17). IR (neat): λ_{max} 3080 (w), 3020 (m), 2980 (s), 2930 (s), 2880 (sh), 2860 (s), 1600 (m), 1495 (w), 1460 (br. m), 1370 (s), 1190 (s), 1180 (s) cm^{-1}.

The 2*R*,3*S* enantiomer and the homologous compounds were prepared by the same method. NMR and IR spectra were all satisfactory.

Preparation of (6Z,9Z)-3,4-Epoxydienes **1a–e.** Me_2CuLi (10 mmol) was prepared from CuI (1.90 g, 10 mmol) and MeLi (1.5 M; 13.3 ml, 20 mmol) in ether (75 ml) at −40°C. The mixture was stirred 30 min, and tosylate **11b** (1.97 g, 4.69 mmol) in ether (10 ml) was added quickly (2 min). The mixture was allowed to warm slowly to −20°C over 1.5 hr, and then was quenched with saturated aq. NH_4Cl (50 ml). The mixture was poured into water (50 ml), the layers were separated, and the aqueous phase was extracted again with ether (2 × 25 ml). The combined organic extracts were washed with water and brine,

dried (Na_2SO_4), and concentrated. The residue was purified by flash chromatography on $AgNO_3$-impregnated silica gel (10%), eluting with 15% ether in hexane, yielding pure **1b** (820 mg, 66%) as a colorless oil. [^{1}H]NMR ($CDCl_3$): δ 5.49 (dtt, 1H, J = 10.7, 7.0, 1.4 Hz; H-7), 5.46–5.35 (m, 2H; H-6, H-10), 5.30 (dtt, 1H, J = 10.7, 7.0, 1.4 Hz; H-9), 2.94 (dt, 1H, J = 6.4, 4.2 Hz; H-4), 2.87 (dt, 1H, J = 6.2, 4.2 Hz; H-3), 2.78 (br t, 2H, J = 6.7 Hz; H-8), 2.39 (m, 1H; H-5), 2.20 (m, 1H; H-5′), 2.03 (dt, 2H, J = 6.8, 6.4 Hz; H-11), 1.65–1.45 (m, 2H; H-2 and H-2′), 1.4–1.2 (m, 12H; H-12 to H-17), 1.04 (t, 3H, J = 7.5 Hz; H-1), 0.86 (t, 3H, J = 6.9 Hz; H-18). IR (neat): λ_{max} 3020 (w), 2985 (m), 2940 (s), 2880 (sh), 2860 (s) cm^{-1}. MS: 264 (M^+, trace), 246 (trace), 235 (0.4), 217 (1.0), 206 (2.9), 192 (11.3), 177 (0.7), 161 (1.0), 147 (2.7), 138 (5.0), 135 (5.1), 121 (9.8), 107 (19.8), 96 (16.4), 95 (27.5), 94 (35.3), 93 (63.7), 91 (40.3), 81 (49.1), 80 (84.4), 79 (100), 78 (33.7), 77 (33.5), 71 (16.5), 69 (31.6), 67 (69.5), 66 (32.7), 59 (41.8), 57 (53.5), 55 (67.3). Elemental analysis of 3*S*,4*R*-**1b**, calcd. for $C_{18}H_{32}O$: C, 81.75; H, 12.20; found: C, 81.71; H, 12.37. 3*R*,4*S*-**1b**: found C, 81.65; H, 12.21.

3*R*,4*S*-**1b** and the homologous diene epoxides were prepared in identical fashion. Yields, NMR, and IR spectra of all compounds were satisfactory. Mass spectra were characterized by small M^+, M-18, and M-29 ions, with moderate intensity diagnostic rearrangement fragments at M-58 (loss of propionaldehyde) and M-72 (loss of 1,2-epoxybutane).

Optical rotations, (3*S*,4*R*) series: **1a** $[\alpha]_D^{23}$ = +2.9° (c = 2.42, CH_2Cl_2); **1b** $[\alpha]_D^{22}$ = +2.3° (c = 2.84, CH_2Cl_2); **1c** $[\alpha]_D^{22}$ = +2.5° (c = 2.70, CH_2Cl_2); **1d** $[\alpha]_D^{23}$ = +2.8° (c = 2.97, CH_2Cl_2); **1e** [α]23 = +2.6° (c = 2.74, CH_2Cl_2). (3*R*,4*S*) series: **1a** $[\alpha]_D^{23}$ = −2.8° (c = 2.45, CH_2Cl_2); **1b** $[\alpha]_D^{22}$ = −2.2° (c = 2.95, CH_2Cl_2); **1c** $[\alpha]_D^{22}$ = −2.1° (c = 2.83, CH_2Cl_2); **1d** $[\alpha]_D^{23}$ = −2.6° (c = 3.12, CH_2Cl_2); **1e** $[\alpha]_D^{23}$ −2.4° (c = 2.92, CH_2Cl_2).

RESULTS

Pheromone Analyses and Field Studies. It had been previously determined that *Probole amicaria* males were attracted by lures containing 6*Z*,9*Z*-*cis*-3,4-epoxy-19:H (Millar et al., 1990), and the data suggested that the addition of 3*Z*,9*Z*-*cis*-6,7-epoxy-19: H to the baits synergized the attraction. An initial experiment was set out to determine if the suspected synergism could be verified and to determine which of the two enantiomers of 3*Z*,9*Z*-*cis*-6,7-epoxy-19:H was responsible for the synergistic effects. Baits contained racemic 6*Z*,9*Z*-*cis*-3,4-epoxy-19:H, alone or in combination with varying amounts of enantiomers of 3*Z*,9*Z*-*cis*-6,7-epoxy-19:H. Two conclusions can be drawn from the data (Table 1). First, additions of small amounts (1–20% of total bait) of

TABLE 1. EFFECT OF ADDING 3Z,9Z-*cis*-6,7-epoxy-19:H ENANTIOMERS[a] TO LURES CONTAINING 6Z,9Z-*cis*-3,4-epoxy-19:H ON MOTH CAPTURES

Lure composition (μg)	Total males captured[b]	
	Probole amicaria	*Anavitrinella pampinaria*
6Z,9Z-*cis*-3,4-epoxy-19:H (500)	32bc	3bc
6Z,9Z-*cis*-3,4-epoxy-19:H (500) ± 3Z,9Z,6*R*,7*S*-epoxy-19:H (5)	90a	0
6Z,9Z-*cis*-3,4-epoxy-19:H (475) + 3Z,9Z,6*R*,7*S*-epoxy-19:H (25)	80ab	0
6Z,9Z-*cis*-3,4-epoxy-19:H (400) + 3Z,9Z,6*R*,7*S*-epoxy-19:H (100)	88a	1c
6Z,9Z-*cis*-3,4-epoxy-19:H (250) + 3Z,9Z,6*R*,7*S*-epoxy-19:H (250)	22c	0
6Z,9Z-*cis*-3,4-epoxy-19:H (500) + 3Z,9Z,6*S*,7*R*-epoxy-19:H (5)	4c	34a
6Z,9Z-*cis*-3,4-epoxy-19:H (475) + 3Z,9Z,6*S*,7*R*-epoxy-19:H (25)	0	39a
6Z,9Z-*cis*-3,4-epoxy-19:H (400) + 3Z,9Z,6*S*,7*R*-epoxy-19:H (100)	0	6b
6Z,9Z-*cis*-3,4-epoxy-19:H (250) + 3Z,9Z,6*S*,7*R*-epoxy-19:H (250)	0	0

[a] Enantiomeric excess of 3Z,9Z,6*R*,7*S*-epoxy-19:H, 88.3%; 3Z,9Z,6*S*,7*R*-epoxy-19:H, 88.2% ee.
[b] Each treatment replicated three times; traps set out June 21–28, 1985. Values followed by the same letter are not significantly different ($P > 0.05$).

3Z,9Z,6*R*,7*S*-epoxy-19:H to 6Z,9Z-*cis*-3,4-epoxy-19:H synergized the attractive response. Second, small amounts of the opposite enantiomer, 3Z,9Z,6*S*,7*R*-epoxy-19:H, resulted in strong behavioral antagonism, such that 5% or greater additions of this compound to the total bait reduced trap captures to zero.

With the enantiomerically enriched forms of 6Z,9Z-*cis*-3,4-epoxy-19:H in hand, an experiment was set out to determine which of the two enantiomers of 6Z,9Z-*cis*-3,4-epoxy-19:H was attractive, in a two component blend with 3Z,9Z,6*R*,7*S*-epoxy-19:H. The results (Table 2) conclusively demonstrated that

TABLE 2. EFFECT OF 6Z,9Z-*cis*-3,4-epoxy-19:H ENANTIOMER ADDITIONS TO LURES CONTAINING 3Z,9Z-6*R*,7*S*-epoxy-19:H ON *Probole amicaria* CAPTURES[a]

Lure composition (μg)	Total males captured[b]
6Z,9Z-*cis*-3,4-epoxy-19:H (500) + 3Z,9Z,6*R*,7*S*-epoxy-19:H (25)	217a
6Z,9Z,3*S*,4*R*-epoxy-19:H (250) + 3Z,9Z,6*R*,7*S*-epoxy-19:H (25)	221a
6Z,9Z,3*R*,4*S*-epoxy-19:H (250) + 3Z,9Z,6*R*,7*S*-epoxy-19:H (25)	30b

[a] Enantiomeric excess of 3Z,9Z,6*R*,7*S*-epoxy-19:H, 88.3%; 6Z,9Z,3*R*,4*S*-epoxy-19:H, 60% ee; 6Z,9Z,3*S*,4*R*-epoxy-19:H, 71% ee.
[b] Each treatment replicated ($N = 3$), June 28–July 15, 1985. Values followed by the same letter are not significantly different ($P > 0.05$).

6*Z*,9*Z*,3*S*,4*R*-epoxy-19:H is primarily responsible for the attractive response. Two other points also emerge from these data. First, the traps baited with blends containing either the racemic 6*Z*,9*Z*-*cis*-3,4-epoxy-19:H or the enantiomerically enriched 6*Z*,9*Z*,3*S*,4*R*-epoxy-19:H resulted in virtually identical trap catches, indicating that the 6*Z*,9*Z*,3*R*,4*S*-epoxy-19:H had no biological activity either as a synergist or an antagonist.

Second, the opposite enantiomer, 6*Z*,9*Z*,3*R*,4*S*-epoxy-19:H, was enantiomerically enriched to the extent of 60% ee. Thus, the residual 6*Z*,9*Z*,3*S*,4*R*-epoxy-19:H impurity may have been responsible for the relatively low trap captures seen with the enantiomerically enriched 3*R*,4*S* bait.

A final experiment was carried out to determine whether 3*Z*,6*Z*,9*Z*-19:H had any biological activity as a synergist or an attractant. This compound had been previously identified in female pheromone gland extracts, but no significant activity had been attributed to this compound in a field test in which small proportions of 3*Z*,6*Z*,9*Z*-19:H were incorporated into baits containing 6*Z*,9*Z*-*cis*-3,4-epoxy-19:H (Millar et al., 1990).

In the new field test, using a more defined basic bait composed of enantiomerically enriched 6*Z*,9*Z*,3*S*,4*R*-epoxy-19:H and 3*Z*,9*Z*,6*R*,7*S*-epoxy-19:H in a 10:1 ratio, it was found that relatively large proportions of 3*Z*,6*Z*,9*Z*-19:H added to the basic bait did indeed increase trap captures (Table 3). Thus the optimum sex attractant determined so far for males of this species is an 8:10:1 blend of 3*Z*,6*Z*,9*Z*-19:H, 6*Z*,9*Z*,3*S*,4*R*-epoxy-19:H, and 3*Z*,9*Z*,6*R*,7*S*-epoxy-19:H.

Low numbers of *Anavitrinella pampinaria* males were captured during 1984 in several traps that contained 6*Z*,9*Z*-*cis*-3,4-epoxy-19:H, 3*Z*,6*Z*-*cis*-6,7-epoxy-19:H, and 3*Z*,6*Z*,9*Z*-19:H as lure components. It was not until the following year that a highly specific sex attractant composed of 6*Z*,9*Z*-*cis*-3,4-

TABLE 3. EFFECT OF ADDITIONS OF 3*Z*,6*Z*,9*Z*-19:H TO BASIC BAIT COMPOSED OF 6*Z*,9*Z*,3*S*,4*R*-epoxy-19:H (250 μg) + 3*Z*,9*Z*,6*R*,7*S*-epoxy-19:H (25 μg) ON *Probole amicaria* MALE CAPTURES[a]

Lure composition (μg)	Total males captured[b]
Basic bait	178b
Basic bait + 3*Z*,6*Z*,9*Z*-19:H (10)	250ab
Basic bait + 3*Z*,6*Z*,9*Z*-19:H (50)	257ab
Basic bait + 3*Z*,6*Z*,9*Z*-19:H (200)	317a
3*Z*,6*Z*,9*Z*-19:H (200)	2c

[a] Enantiomeric excess of 3*Z*,9*Z*,6*R*,7*S*-epoxy-19:H, 88.3%; 6*Z*,9*Z*,3*S*,4*R*-epoxy-19:H, 71% ee.
[b] Each treatment replicated ($N = 3$) May 29–June 12, 1986. Values followed by the same letter are not significantly different ($P > 0.05$).

epoxy-19:H and 3*Z*,9*Z*,6*S*,7*R*-epoxy-19:H in an approximate ratio of 100:1 to 20:1, was found (Table 1).

A further experiment set out at the end of the flight season, comparing mixtures of the racemic 3,4-epoxide or its enantiomers in combination with the 3*Z*,9*Z*,6*S*,7*R*-epoxy-19:H, gave low overall trap captures, but indicated that the attractive enantiomer was possibly the 3*S*,4*R* epoxide (*cis*-3,4:3*S*,4*R*:3*R*,4*S*; 9:6:0 captures, respectively). We have been unable to obtain female insects, and so could not establish whether these compounds were pheromone components for *A. pampinaria*.

Sicya macularia male moths have been shown to be attracted to blends of 6*Z*,9*Z*-*cis*-3,4-epoxy-19:H with 3*Z*,6*Z*,9*Z*-19:H (Millar et al., 1990). It has also been demonstrated that trace amounts of 3*Z*,9*Z*-*cis*-6,7-epoxy-19:H added to otherwise attractive baits completely suppressed trap captures (Millar et al., 1990). An experiment was set out to determine which of the two 6*Z*,9*Z*-*cis*-3,4-epoxy-19:H enantiomers was attractive and which of the two enantiomers of 3*Z*,9*Z*-*cis*-6,7-epoxy-19:H was the behavioral antagonist (Table 4). The first part of the experiment was not successful, as there were no significant differences ($P = 0.05$) between lures containing racemic or enantiomerically enriched 3,4-epoxides (significance level between second and first treatments, 1985 data set, was $P = 0.06$).

In the second part of the experiment, both enantiomers of the 6,7-epoxide were powerful behavioral antagonists, as addition of either enantiomer at a concentration of only 1% of the total bait completely suppressed attraction (Table 4).

TABLE 4. CAPTURE OF *Sicya macularia* MALES IN TRAPS BAITED WITH 3*Z*,6*Z*,9*Z*-19:H AND C_{19}-MONOEPOXYDIENE ENANTIOMERS[a]

Lure composition (μg)	Total males captured[b] 1985	1986
6*Z*,9*Z*-*cis*-3,4-epoxy-19:H (450) + 3*Z*,6*Z*,9*Z*-19:H (50)	9ab	6b
6*Z*,9*Z*,3*S*,4*R*-epoxy-19:H (225) + 3*Z*,6*Z*,9*Z*-19:H (50)	31a	27a
6*Z*,9*Z*,3*R*,4*S*-epoxy-19:H (225) + 3*Z*,6*Z*,9*Z*-19:H (50)	7b	11ab
6*Z*,9*Z*-*cis*-3,4-epoxy-19:H (450) + 3*Z*,6*Z*,9*Z*-19:H (50) + 3*Z*,9*Z*,6*S*,7*R*-epoxy-19:H (5)	0	0
6*Z*,9*Z*-*cis*-3,4-epoxy-19:H (450) + 3*Z*,6*Z*,9*Z*-19:H (50) + 3*Z*,9*Z*,6*R*,7*S*-epoxy-19:H (5)	0	0

[a] Enantiomeric excess of 3*Z*,9*Z*,6*R*,7*S*-epoxy-19:H, 88.3%; 3*Z*,9*Z*,6*S*,7*R*-epoxy-19:H, 88.2% ee; 6*Z*,9*Z*,3*R*,4*S*-epoxy-19:H, 60% ee; 6*Z*,9*Z*,3*S*,4*R*-epoxy-19:H, 71% ee.

[b] Each treatment replicated; August 21–September 10, 1985 ($N = 4$) and July 14–31 1986 ($N = 3$). Values followed by the same letter are not significantly different ($P > 0.05$).

It should also be mentioned that the traps that contained the 6*S*,7*R* enantiomer captured *A. pampinaria* males but those with the 6*R*,7*S*-epoxide took none, supporting the data in Table 1.

A high degree of enantiomeric selectivity appeared to be involved in the attraction of males of another geometrid, *Lycia ursaria*. Of a total of 20 specimens captured in binary baited traps in 1986, 16 were taken in traps baited with mixtures of 3*Z*,6*Z*,9*Z*-19 : H and 6*Z*,9*Z*,3*S*,4*R*-epoxy-19 : H, one with each of the corresponding C_{18} and C_{20} blends and two with a mixture of C_{19} triene hydrocarbon and 3*R*,4*S*-epoxydiene. No males were taken in traps that contained the foregoing compounds as single component lures, and ternary-baited survey traps were not set out. The field-trapping data correlated well with antennal responses in GC-EAD of a standards mixture. In studies with synthetic 3*Z*,6*Z*,9*Z*-trienes and monoepoxide analogs of 3*Z*,6*Z*,9*Z*-trienes and 6*Z*,9*Z*-dienes, the greatest antennal stimulation was elicited by 6*Z*,9*Z*-*cis*-3,4-epoxy-19 : H. Appreciable, although lower, stimulation occurred in response to 3*Z*,9*Z*-*cis*-6,7-epoxy-19 : H, to the 3,4- and 6,7- C_{18} and C_{20} epoxides and to 3*Z*,6*Z*,9*Z*-19 : H.

Field tests with sex attractant baits containing C_{17} epoxides in combination with 3*Z*,6*Z*,9*Z*-17 : H revealed that two species from the tribe Semiothisini and the genus *Itame* exhibited a high degree of enantiomeric specificity in their behavioral responses. A total of 25 *I. brunneata* males were captured during 1986, and of these, 20 were taken in traps baited with 6*Z*,9*Z*,3*S*,4*R*-epoxy-17 : H as a single component or as the major component together with 3*Z*,6*Z*,9*Z*-17 : H; the remainder were captured in traps baited with 10 : 1 6*Z*,9*Z*,3*S*,4R-epoxy-18 : H + 3*Z*,6*Z*,9*Z*-18 : H. As an indication of the specificity of these captures, approximately 400 differently baited traps were routinely set out at any one time in the trapping area, and no males of this species were captured in traps other than those indicated.

Males of *I. occiduaria*, which cooccur in this region with *I. brunneata*, were captured by a 9 : 1 blend of 6*Z*,9*Z*,3*R*,4*S*-epoxy-17 : H and 3*Z*,6*Z*,9*Z*-17 : H (Table 5). It would appear that the attractive responses of males of *I. brunneata* and *I. occiduaria* are suppressed by high concentrations of the "opposite" enantiomer, as neither species was captured by traps baited with 6*Z*,9*Z*-*cis*-3,4-epoxy-17 : H as a single component or in blends with 3*Z*,6*Z*,9*Z*-17 : H. Very large EAG responses were recorded from antennae of male *I. occiduaria*, with the largest responses (ca. 10 mV) being elicited by both enantiomers of 6*Z*,9*Z*-*cis*-3,4-epoxy-17 : H and by 3*Z*,6*Z*,9*Z*-17 : H (ca. 7 mV).

Male moths of a third species in the tribe Semiothisini, *Epelis truncataria*, were captured in all survey traps (replicated twice) that contained 6*Z*,9*Z*-*cis*-3,4-epoxy-17 : H or its enantiomers as single components or as 10 : 1 blends with 3*Z*,6*Z*,9*Z*-17 : H (Table 6). Trap captures were greater with racemic 6*Z*,9*Z*-*cis*-3,4-epoxy-17 : H than with the analogous enantiomerically enriched com-

TABLE 5. ATTRACTANCY OF BLENDS CONTAINING 6Z,9Z-*cis*-3,4-epoxy-17:H OR ITS ENANTIOMERS[a] AND 3Z,6Z,9Z-17:H FOR MALES OF TWO *Itame* SPECIES

Lure composition (μg)	Total males captured[b]	
	I. occiduaria	*I. brunneata*
6Z,9Z-*cis*-3,4-epoxy-17:H (450) + 3Z,6Z,9Z-17:H (50)	0	0
6Z,9Z,3*R*,4*S*-epoxy-17:H (225) + 3Z,6Z,9Z-17:H (50)	56	0
6Z,9Z,3*S*,4*R*-epoxy-17:H (225) + 3Z,6Z,9Z-17:H (50)	0	5

[a] Enantiomeric excess of 6Z,9Z,3*R*,4*S*-epoxy-17:H, 69%; 6Z,9Z,3*S*,4*R*-epoxy-17:H, 69% ee.
[b] Each treatment replicated ($N = 3$) July 14–August 12, 1986.

TABLE 6. CAPTURES OF *Epelis truncataria* AND *Semiothisa signaria dispuncta* MOTHS IN 1986 SURVEY TRAPS BAITED WITH 3Z,6Z,9Z-17:H AND RACEMIC OR ENANTIOMERIC[a] 6Z,9Z-*cis*-3,4-epoxy-17:H

Lure composition (μg)	Total males captured[b]	
	E. truncataria	*S. signaria*
6Z,9Z-*cis*-3,4-epoxy-17:H (500)	226a	13a
6Z,9Z,3*S*,4*R*-epoxy-17:H (500)	64bc	0
6Z,9Z,3*R*,4*S*-epoxy-17:H (500)	76bc	2a
6Z,9Z,3*S*,4*R*-epoxy-17:H (500) + 6Z,9Z,3*R*,4*S*-epoxy-17:H (50)	106abc	0
6Z,9Z,3*S*,4*R*-epoxy-17:H (50) + 6Z,9Z,3*R*,4*S*-epoxy-17:H (500)	117abc	1a
6Z,9Z,*cis*-3,4-epoxy-17:H (500) + 3Z,6Z,9Z-17:H (50)	129abc	44a
6Z,9Z,3*S*,4*R*-epoxy-17:H (500) + 3Z,6Z,9Z-17:H (50)	170ab	0
6Z,9Z,3*R*,4*S*-epoxy-17:H (500) + 3Z,6Z,9Z-17:H (50)	43c	2a

[a] Enantiomeric excess of 6Z,9Z-3*R*,4*S*-epoxy-17:H, 69%; 6Z,9Z,3*S*,4*R*-epoxy-17:H, 69% ee.
[b] Total number of males captured in replicated treatments ($N = 2$) between May 20 and June 19, 1986. Values followed by the same letter are not significantly different ($P > 0.05$).

pounds. Considerable variability in trap captures occurred within some of the twice-replicated treatments, possibly because of the diversity in vegetation throughout the trapping region, or differences in vegetation from which the traps were hung. The data indicated that *E. truncataria* males may respond to both epoxide enantiomers and do not discriminate between them, a response rarely experienced in our field tests with the epoxides.

Male antennal responses to C_{17-22} 6Z,9Z- and 3Z,6Z,9Z-unsaturated hydrocarbons and their derived monoepoxides were studied by GC-EAD. Significant antennal responses were elicited only by 6Z,9Z-*cis*-3,4-epoxy-17:H and 3Z,9Z-

cis-6,7-epoxy-17:H. When the antennal responses were measured using an electroantennograph, the largest responses were elicited by 6*Z*,9*Z*-*cis*-3,4-epoxy-17:H (~4.7 mV), 6*Z*,9*Z*,3*S*,4*R*-epoxy-17:H (~5.1 mV) and 6*Z*,9*Z*,3*R*,4*S*-epoxy-17:H (~4.1 mV); the next most stimulatory compounds were 3*Z*,9*Z*,6*S*,7*R*-epoxy-17:H (~2.1 mV) and 3*Z*,9*Z*,6*R*,7*S*-epoxy-17:H (~1.7 mV), in accord with the GC-EAD responses; all other compounds tested gave responses near background.

It had been demonstrated previously that male *Semiothisa signaria dispuncta* moths were attracted by blends of 3*Z*,6*Z*,9*Z*-17:H and 6*Z*,9*Z*-*cis*-3,4-epoxy-17:H, both of which had been found in female pheromone gland extracts (Millar et al., 1987). Survey trap data from 1986 (Table 6), although only replicated twice, suggested that the two enantiomers of 6*Z*,9*Z*-*cis*-3,4-epoxy-17:H may act synergistically, because the traps baited with racemic epoxide caught 13 specimens, while the corresponding traps baited with the enantiomerically enriched isomers caught none. It was not possible to draw firm conclusions from the data because of the low number of replicates.

We had previously reported (Millar et al., 1987) that male *Semiothisa ulsterata* moths were attracted to 3*Z*,6*Z*-*cis*-6,7-epoxy-17:H. An experiment set out in 1986 conclusively demonstrated that 3*Z*,9*Z*,6*S*,7*R*-epoxy-17:H was the attractive enantiomer (Tables 7 and 8). The 6*R*,7*S* enantiomer neither synergized nor antagonized the attractive response, as traps baited with either 3*Z*,9*Z*,6*S*,7*R*-epoxy-17:H or the racemic 3*Z*,9*Z*-*cis*-6,7-epoxy-17:H were equally attractive (Table 7).

A further experiment was set out to test whether the attractive response of *S. ulsterata* males was antagonized by 3*Z*,6*Z*,9*Z*-17:H. This compound had not been found in abdominal tip extracts of conspecific females, but male antennae stimulated with this compound did give a considerable EAG response (Millar et al., 1987). Thus, 3*Z*,9*Z*,6*S*,7*R*-epoxy-17:H was put out in blends with 3*Z*,6*Z*,9*Z*-17:H, keeping the total weight of test compounds at 500 μg/bait.

TABLE 7. ATTRACTANCY OF RACEMIC OR CHIRAL[a] 3*Z*,9*Z*-*cis*-6,7-epoxy-17:H FOR *Semiothisa ulsterata* MALES

Lure composition (μg)	Total males captured[b]
6*Z*,9*Z*-*cis*-6,7-epoxy-17:H (500)	85a
3*Z*,9*Z*,6*S*,7*R*-epoxy-17:H (250)	67a
3*Z*,9*Z*,6*R*,7*S*-epoxy-17:H (250)	1b

[a] Enantiomeric excess of 3*Z*,9*Z*,6*R*,7*S*-epoxy-17:H, 88.3%; 3*Z*,9*Z*,6*S*,7*R*-epoxy-17:H, 88.2% ee.
[b] Each treatment replicated ($N = 3$) June 10–20, 1986. Values followed by the same letter are not significantly different ($P > 0.05$).

TABLE 8. EFFECT OF BLENDS OF 3Z,6Z,9Z-17:H WITH 3Z,9Z,6S,7R-epoxy-17:H[a] ON ATTRACTION OF *Semiothisa ulsterata* MALE MOTHS

Lure composition (μg)	Total males captured[b]
3Z,9Z,6S,7R-epoxy-17:H (500)	99a
3Z,9Z,6R,7S-epoxy-17:H (500)	2c
3Z,9Z,6S,7R-epoxy-17:H (475) + 3Z,6Z,9Z-17:H (25)	59b
3Z,9Z,6S,7R-epoxy-17:H (400) + 3Z,6Z,9Z-17:H (100)	44b
3Z,9Z,6S,7R-epoxy-17:H (250) + 3Z,6Z,9Z-17:H (250)	7c
3Z,9Z,6S,7R-epoxy-17:H (100) + 3Z,6Z,9Z-17:H (400)	2c

[a] Enantiomeric excess of 3Z,9Z,6S,7R-epoxy-17:H, 88.2% ee.
[b] Each treatment replicated ($N = 3$) June 23–July 10, 1986. Values followed by the same letter are not significantly different ($P > 0.05$).

Significant suppression of trap catches occurred with the two-component baits (Table 8), indicating that 3Z,6Z,9Z-17:H was indeed a behavioral antagonist for this species. The analogous series of baits with the nonattractive 6R,7S enantiomer gave no captures at all (data not shown).

Stereoselective Synthesis of 6Z,9Z-cis-3,4-Epoxides. The chiral epoxides **1a–e** were synthesized as shown in Scheme 1. Thus, the inexpensive 2-butyn-1,4-diol **2,** on treatment with 1 equivalent of dihydropyran, gave a moderate yield (40%) of the monoprotected alcohol **3.** This was quantitatively converted to the tosylate **4** by reaction with tosyl chloride and powdered KOH in ether at −10°C (Brandsma, 1971).

The 1,4-diynes **6** were prepared as previously described (Millar and Underhill, 1986b) by treatment of the Grignard reagents formed from alkynes **5** with propargyl bromide at −10°C to +20°C, with CuI catalysis (74–84%). CuI-catalyzed reaction of the alkynylmagnesium bromides prepared from diynes **6** with tosylate **4** in THF at −10°C to 20°C (Verkruijsse and Hasselar, 1979) gave triynes **7.** Removal of the THP protecting group in acidified methanol yielded triynols **8,** which were conveniently purified by recrystallization from hexane (65–85% from **4**). Hydrogenation over Lindlar's catalyst gave the trienols **9** (79–87%), which were then subjected to asymmetric epoxidation (Katsuki and Sharpless, 1980), yielding the chiral epoxyalcohols **10** (52–81%). The enantiomeric excess obtained in the asymmetric induction was determined by derivatization of the epoxyalcohols **10** with (2S)-2-acetoxypropionyl chloride, and capillary GC of the resulting derivatives (Doolittle and Heath, 1984; Slessor et al., 1985). Baseline separation of the diastereomers formed from **10a–d** was obtained on a DB-210 capillary GC column (30 m × 0.25 mm). The derivatives from **10e** were not completely resolved.

Tosylation of the epoxyalcohols **10** (71–100%), followed by chemoselective alkylation of the resulting tosylates **11** with dimethyl lithium cuprate in

ether at −40°C to −20°C (Mori and Ebata, 1981, 1986) completed the syntheses, yielding the chiral diene epoxides **1** (52–67%) in >98% chemical and geometric isomeric purity after purification on $AgNO_3$ on silica gel and Kugelrohr distillation. Enantiomeric purities varied from 57% to 90% enantiomeric excess. Overall yields for the syntheses were ~15%.

DISCUSSION

The general trend of the results reported here indicates that behavioral responses of the various moths usually are associated with one enantiomer, or a preponderance of one enantiomer. However, it must be emphasized that the trapping data were obtained using enantiomerically enriched, not enantiomerically pure, compounds. Where chirality appears to be a factor in attraction or behavioral antagonism, we cannot rule out the possibility that any one set of results may be due in part to the nonracemic blend of enantiomers in the enantiomerically enriched materials.

There were indications in the study by Wong et al. (1985) that multiple chiral and regioisomeric components could be involved in lepidopteran sex attractants and sex pheromones. However, to our knowledge, the sex attractant blend of *P. amicaria* represents the first example in the Lepidoptera of a blend involving two chiral components and the first example of a blend of two regioisomers. The opposite enantiomer of the attractive 3,4-epoxide and either enantiomer of the 9,10-epoxide regioisomer (Millar et al., 1990) are apparently benign, acting neither as synergists nor as antagonists, while the opposite enantiomer (3*Z*,6*Z*,6*S*,7*R*-O-19:H) of the attractive 6,7-epoxide is a strong behavioral antagonist (Table 1).

3*Z*,6*Z*,9*Z*-19:H has been identified in female abdominal tip extracts (Millar et al., 1990), and elicited significant increases in trap captures when incorporated into baits. The triene is presumably present as a pheromone precursor and also has a role as a pheromone component. Thus, the optimal attractant is a blend of chiral regioisomers (6*Z*,9*Z*,3*S*,4*R*-epoxy-19:H + 3*Z*,9*Z*,6*R*,7*S*-epoxy-19:H), in combination with 3*Z*,6*Z*,9*Z*-19:H.

A sympatric species, *A. pampinaria*, is attracted by the opposite enantiomer of the 6,7-epoxide regioisomer (3*Z*,9*Z*,6*S*,7*R*-epoxy-19:H) in combination with 6*Z*,9*Z*-*cis*-3,4-epoxy-19:H (Table 1). Attraction to field baits is strongly suppressed by the presence of the 6*R*,7*S* epoxide in the sex attractant blend for *P. amicaria*. Thus, even though the ratio of the two regioisomers in the attractive blends for each species is approximately the same (10:1), the chirality of the two regioisomers is the critical factor in determining the attraction of each species.

A third species, *S. macularia*, was attracted to traps baited with mixtures

of 3Z,6Z,9Z-19:H and 6Z,9Z,3*S*,4*R*-epoxy-19:H. These compounds were mutually synergistic. Attraction was completely suppressed by addition of 1% of either enantiomer of the 3Z,6Z-*cis*-6,7-epoxy-19:H, preventing this insect from being cross-attracted to the sex attractants for either *P. amicaria* or *A. pampinaria*.

The strong behavioral antagonism by either enantiomer raises two possibilities. First, as the 6,7-epoxide enantiomers were only approximately 94% enantiomerically pure (enantiomeric excesses of >88%, Millar and Underhill, 1986a), complete inhibition may have resulted from the trace amounts of enantiomeric impurity (6 parts in 10,000 of the total bait), indicating extraordinary sensitivity in the detection and processing of the chemical signal.

The second, and more intriguing possibility, is that the male insect may possess either one set of antennal receptors that will accept either enantiomer of the inhibitory compound equally well or that it has two types of antennal receptors, each of which is specific for one enantiomer. There is precedent for the latter case, as male gypsy moth antennae have been shown to possess two types of sensory cells, each of which is specifically tuned to one of the two enantiomers of the chiral epoxide pheromone, disparlure (Hansen, 1984).

The response of the insect also is suppressed significantly by somewhat larger amounts of 3Z,6Z-*cis*-9,10-epoxy-19:H (Millar et al., 1990). Thus the responses of male *S. macularia* are characterized both by the specificity of the attractant blend and by sensitivity to a number of behavioral antagonists.

Data on a fourth species, *L. ursaria*, indicated that this insect was attracted by synergistic mixtures of 3Z,6Z,9Z-19:H + 6Z,9Z,3*S*,4*R*-epoxy-19:H, the same components that formed the most attractive mixtures for the previous species, *S. macularia*. Chirality was obviously critical, as only one insect was attracted to the blend containing the other enantiomer. Reasons for the low overall trap captures are not clear. There may be one or more missing components, as suggested by GC-EAD studies, in which several similar compounds elicited significant responses from male antennae. Alternatively, and possibly concurrently, the enantiomeric impurity in the enantiomerically enriched 6Z,9Z,3*S*,4*R*-epoxy-19:H used in the baits may have been behaviorally antagonistic. No females of this species have been obtained, so it has not been possible to obtain any clues as to the actual pheromone blend for this insect.

Within the tribe Semiothisini of the subfamily Ennominae, the two *Itame* species, the two *Semiothisa* species, and *E. truncataria* all produced and/or were attracted by baits composed of one or more C_{17} compounds. The two *Itame* species were attracted to two-component blends of 3Z,6Z,9Z-17:H with one of the enantiomers of the corresponding 3,4-epoxide, with each species using a different enantiomer. Trap captures of each species were suppressed by the presence of the other enantiomer. A third species, *Semiothisa signaria dispuncta*, was attracted by blends of the racemic 6Z,9Z-*cis*-3,4-epoxy-17:H with

3Z,6Z,9Z-17:H. Although all three species share common components in their blends, a subtle interplay of chiral attractants and behavioral antagonists limits cross-attraction.

The interpretation of the trap captures and GC-EAD responses for the fourth species in the tribe Semiothisini, *E. truncataria*, is not altogether clear. Certainly, the insect is highly attracted to one or both enantiomers of 6Z,9Z-*cis*-3,4-epoxy-17:H (Table 6). However, because of the high variability in the trap captures for individual baits, the effects of individual enantiomers or of addition of the 3Z,6Z,9Z-17:H cannot be determined at this stage.

In addition, males of both *E. truncataria* and *Semiothisa signaria dispuncta* (Walker) respond to blends of 6Z,9Z-*cis*-3,4-epoxy-17:H + 3Z,6Z,9Z-17:H. In this region, the flight periods of these two species overlap extensively. The mechanism of their premating isolation is likely based upon factors other than common pheromone components, such as additional pheromone components or antagonists, or different temporal windows for calling and flight activity.

It is interesting also to note that several of the *Semiothisini* spp. were attracted quite strongly in the field to homologs of the best attractants (data not shown, and Millar et al., 1987). Whereas it is fairly common to see some response to homologs in EAG and GC-EAD studies, in our experience, homologs are not often very successful field attractants. This suggests that there has been little selection pressure on male insects in this tribe to discriminate accurately on the basis of chain length; that is, there are apparently few sympatric species using C_{16} or C_{18} trienes or monoepoxydienes in their pheromone blends. This hypothesis is supported by field trials, as we have not discovered any lepidopteran sex attractants during field surveys with the C_{16} analogs of the trienes and epoxides discussed here, and only one sex attractant blend in field surveys with C_{18} compounds (Millar and Underhill, manuscript in preparation).

Chirality has been recognized as a factor in inter- and intraspecific pheromone communication for a number of years. In the family Lepidoptera, however, there are few reported instances of chiral compounds in the many hundred pheromone blends now known (Arn et al., 1986). Bell and Meinwald (1986) provided an excellent review of these chiral pheromones. The work reported here and recent work with unsaturated epoxides from our laboratory (Wong et al., 1985; Millar and Underhill, 1986a; Millar et al., 1987) and other groups (Descoins and Frérot, 1984; Bell and Meinwald, review to 1986) has shown that unsaturated epoxides are components of attractant or pheromone blends for more than 30 lepidopteran species and demonstrates that chirality in lepidopteran pheromones may indeed be fairly widespread. Certainly the potential is there, in terms of the possibilities for induction of chirality into substrates by straightforward biochemical modifications such as epoxidation of unsaturated achiral substrates. Indeed, enzymatic transformations producing quaternary car-

bons substituted with four different groups would generally be expected to produce chiral rather than racemic products.

In analogy to the major class of lepidopteran pheromones comprised of unsaturated aldehydes, alcohols, and acetates, structural variation in the triene/epoxide class of pheromones provides a mechanism by which discrete pheromone communication channels can be maintained. However, the types of structural variations in this new class are somewhat different. Structural variations that we now know of include chain length (C_{17-23}), degree of unsaturation (one to four double bonds), position of the epoxides (3,4; 6,7; 9,10), and enantiomeric composition of the epoxides (*R,S* or *S,R* for any one of the regioisomers). There has been one report of a related ketone (6*Z*,9*Z*-nonadecadien-3-one) as a pheromone component for the geometrid *Peribatodes rhomboidaria* (Buser et al., 1985). It also is interesting that there appear to be a number of constraints on the structural variation possible. In the published data on approximately 30 species, and in the unpublished data that we have on approximately 60 more species (Millar and Underhill, manuscripts in preparation), the core double bonds are always in the 3, 6, or 9 positions, and the core double bonds are always *cis*. Several species produce tetraenes, with the additional double bond in either the 1 position (Connor et al., 1980; Bestmann et al., 1982; Roelofs et al., 1982; Underhill et al., 1987) or the 11 position (Wong et al., 1985). There has also been one report of 1,3*E*,6*Z*,9*Z*-19:H antagonizing the response of the Bruce spanworm, *Operophtera bruceata*, to its pheromone, 1,3*Z*,6*Z*,9*Z*-19:H (Underhill et al., 1987). In analogous fashion, the epoxides, which are presumably derived from the unsaturated hydrocarbon precursors (Roelofs and Bjostad, 1984), are always in the 3, 6, or 9 position, and they are always *cis* epoxides. While these structural generalizations may not survive the test of time, they have proven to be very useful and highly successful in predicting the types of compounds that may be attractants or pheromones for speculative synthesis and field testing.

The efficiency and conservativeness of the system is obvious. A single 3*Z*,6*Z*,9*Z*-triene hydrocarbon substrate, submitted to a single regio- and enantiospecific biochemical epoxidation, can yield any one of six different isomers, assuming that the geometry of the epoxidized double bond is retained. If two epoxide isomers are combined, there are fifteen different possible combinations, irrespective of other factors limiting attractancy, such as blend ratio, chain length, removal or addition of a double bond, or the presence of inhibitors. With three or more components, the number of possible combinations and the specificity of attraction becomes very high indeed.

Synthesis of Attractants. Our initial synthetic strategy involved a disconnection between C_5 and C_6 of the epoxides **1,** giving a chiral 5-carbon epoxide fragment that was common to all the homologs, and a diene anion equivalent of variable chain length. We (Millar and Underhill, 1986a) and others (Nicolaou et al., 1984) have used a coupling of this type previously in preparing

homoallylic epoxides. Either enantiomer of the 5-carbon epoxide was readily available from Sharpless asymmetric epoxidation (Rossiter et al., 1981) of 2Z-penten-1-ol, followed by conversion of the alcohol to a leaving group, while diene anions of variable length could be prepared by metallation of 1-bromo-1Z,4Z-dienes, in turn available from 1,4-diynes by sequential bromination of terminal alkynes and reduction. This route had the advantage of converging at the final step, a key point to consider in the efficient synthesis of a homologous series of compounds.

In practice, this route was beset by a number of technical problems. First, the isolated yields of the small water-soluble 2,3-epoxypentanols from the asymmetric epoxidation were moderate (~50%), as had been previously reported (Rossiter et al., 1981). Second, the 1-bromo-1Z,4Z-dienes required for the coupling step proved difficult to purify in quantity, as the compounds partially decomposed on distillation and were not separated from impurities by flash chromatography on silica gel. Third, conversion of the diene bromides to alkenylmagnesium bromides gave impurities that rapidly quenched the CuI catalyst in the coupling step. In consequence, we reluctantly abandoned this route in favor of a more straightforward but less convergent route (Scheme 1).

This synthesis was also not without problems. The enantiomeric excesses of several of the epoxides **10** were found to be considerably less than anticipated. The cause of this was eventually determined to be an incompletely dried batch of methylene chloride that had been used in several of the asymmetric epoxidations. At this stage, it was too late to resynthesize the precursors without missing part of the field season, so the syntheses were completed using the materials on hand, despite their lowered enantiomeric excesses.

In addition, triynol **8d** proved to be intractable to catalytic reduction, probably because of trace impurities that poisoned the catalyst. In consequence, the THP protected precursor **7d** was reduced with dicyclohexylborane (Brown, 1975) to the triene, followed by removal of the THP group to give trienol **9d**.

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SEXUAL BEHAVIOR OF *Matsucoccus josephi* (HOMOPTERA: MARGARODIDAE) Asynchronous Adult Male Emergence and Release of Female Sex Pheromone[1]

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Abstract—The daily emergence patterns of *Matsucoccus josephi* adults and third-instar male larvae raised on artificially infested saplings of *Pinus halepensis* were determined. A single peak of emergence was found for adult males between 0300 and 0500 hr. Adult females emerged throughout the day, with maximum emergence between 0500 and 0700 hr. Two daily emergence periods were observed in third-instar male larvae, one between 0300 and 0900 hr and the other between 1700 and 2100 hr. Airborne pheromone emitted by adult virgin females was collected using a flow system. No significant differences were recorded in the attraction of the male to crude pheromone collected at different times of day. A single female, virgin or half an hour after mating, was sufficient to attract the males in an olfactometer system. From minor differences in pheromone release throughout the day, and from the lesser degree of attraction by females half an hour after mating, it is assumed that there is no mechanism controlling the daily release of the female sex pheromone. Reduction of pheromone emission after mating is suggested.

Key Words—*Matsococcus josephi*, Homoptera, Margarodidae, sexual behavior, pheromone release.

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INTRODUCTION

The Israeli pine bast scale *Matsucoccus josephi* Bodenh. and Harpaz is the most noxious pest of *Pinus halepensis* Mill. and *P. brutia* ssp. *eldarica* (Medw.) Nahal in Israel (Mendel, 1987). The scale occurs on all aboveground parts of the tree. During feeding it secretes a poisonous saliva that disrupts the water transport in the tree and frequently causes its death (Mendel and Liphschitz, 1988). In its latent phase the scale population occurs mainly on the partly scaly bark section of the stem, whereas during the epidemic phase it infests most of the shoots (Golan et al., 1983).

The feeding stage of the male in the genus *Matsucoccus* is shorter than that of the female. At the end of the feeding period the male develops into a third-instar larva that leaves the feeding spot to look for a pupation site, the forest floor in the case of *M. josephi* (Bodenheimer and Neumark, 1955; Mendel et al., 1989). The emergence of the adult males is synchronous with that of the adult females and is usually related to the temperature and its effect on the pine host (Mendel et al., 1990; Riom and Fabre, 1977). Like several other species of *Matsucoccus*, the adult males emerge from the litter to fly or walk on the stem and crown in search of emerging females (e.g., Golan et al., 1983; McKenzie, 1942; Riom and Fabre, 1977; Unruh, 1985). Most known species are attracted to the female sex pheromone, a phenomenon first recorded by Doane (1966) in the males of the red pine scale, *M. resinosae* Bean and Godwin. Evidence of its presence has since been demonstrated in several *Matsucoccus* spp. (Sternlicht and Dunkelblum, unpublished; Young and Qi, 1983; Young et al., 1984; Park et al., 1986) and was identified for *M. resinosae*, *M. matasummurae*, and *M. thumbergianae* (Lanier et al., 1989).

The biology and morphology of males and females of the more common *Matsucoccus* species (Beardsley, 1968; Bodenheimer and Neumark, 1955; Boratinski, 1952; Ray, 1982; Siewniak, 1976) and male morphology of several fossil species (Koteja, 1984) are known. The adult males live about half a day (Beardsley, 1968; Mendel et al., 1989; Siewniak, 1976; Riom and Fabre, 1977) and females for several days and even several weeks (e.g., Bodenheimer and Neumark, 1955; Riom and Fabre, 1977). Females may be inseminated during the first 10 days after emergence (Bodenheimer and Neumark, 1955). However, little is known about the daily emergence pattern of the adults and their sexual behavior.

The objectives of the present study were to elucidate several unfamiliar aspects of the reproductive biology of *Matsucoccus:* the release pattern of sex pheromone in *M. josephi,* and the emergence pattern of the adults. This provided also the opportunity to study the emergence pattern of third-instar male larvae.

METHODS AND MATERIALS

Emergence Pattern. The emergence pattern of adult females and third-instar male larvae was determined by collections from 15 two-year-old saplings of *P. halepensis* growing in black polythene bags and infested with *M. josephi* ovisacs a month earlier. Each sapling was placed into a plastic tray (40 × 30 × 10 cm) lined with a sheet of paper. The emerging insects were removed from the plant or the tray with a fine brush and counted. The emergence pattern of the adult males was determined by counting the individuals emerging from masses of pupae collected from saplings and others kept in Petri dishes. The plants were maintained in a greenhouse under natural light with continuous temperature recording (Figure 1). Emerging scales were then collected at 2-hr intervals for three days, beginning at noon on June 29.

Airborne Pheromone Collection. Airborne pheromone emitted by adult virgin females of *M. josephi* was collected using a flow system. The scales were obtained from a mass rearing (Mendel et al., 1989). Two systems (A and B) were operated. In each system the females were maintained on Whatman No. 1 filter paper in three glass tubes closed at both ends with a copper wire screen. The tubes were placed in a glass cartridge. The cartridge was installed between a U-shaped tube containing active charcoal to purify the incoming air and a 50–80 mesh Porapak Q column (130 mm long × 10 mm diam. containing 1 g adsorbent) to trap the pheromone. The Porapak Q trap was connected to a small pump (Charles Austin Pumps, Weybridge, England) through a Dwyer flow meter equipped with a control valve. Air was drawn through the system at a rate of 1 liter/min. No significant breakthrough of pheromone was observed in this system. This was shown in an earlier experiment by adding a second Porapak Q column in line; bioassays proved that almost all active material was trapped in the first column (Z. Mendel and E. Dunkelblum unpublished). On July 29, system A was operated with 473 females, consisting of two groups: the first of 300 scales that had emerged after midnight and mainly during the early morning, collected around 0700 hr, and the second consisting of 173 females collected the previous morning (July 28). They were stored for 24 hr at 6°C. System B contained 321 tested females that had emerged in the early morning and were collected around 0700 hr. System A was operated for three days from 0330 hr on July 29 until 1230 hr on August 1. System B was operated for two days from 0830 hr on July 30 to 1230 hr on August 1. The Poropak column in each system was changed every 4 hr. Both systems were run at 26 ± 2°C under natural light. Lack of ovisacs indicated that the females were virgin. Every 4-hr collection was considered as a sample unit.

The adsorbed pheromone was eluted from the Porapak Q traps with 8 ml

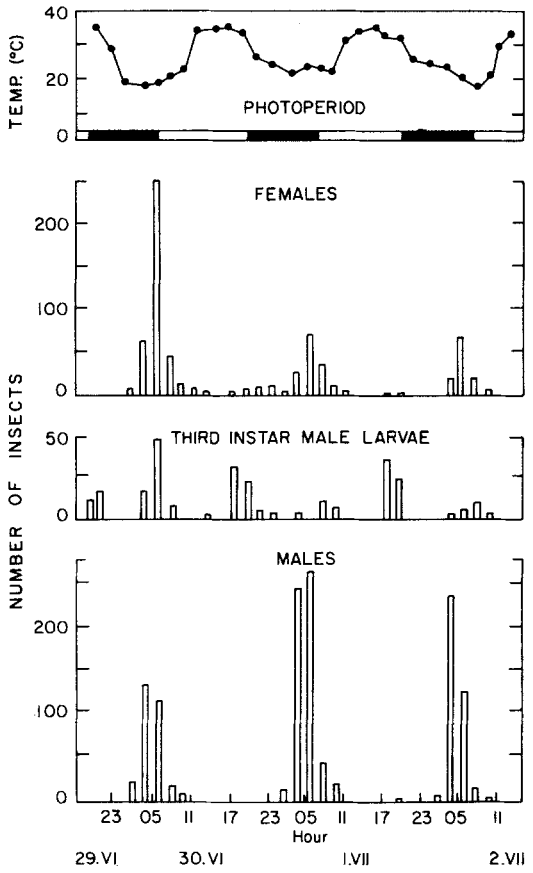


FIG. 1. Daily emergence pattern of adult females, adult males, and third-instar male larvae of *Matsucoccus josephi* in relation to the thermoperiod and the photoperiod (black: darkness).

distilled diethyl ether until 5 ml extract was collected. One milliliter of hexane was added to the ether extract, and the solution was concentrated to 1 ml at room temperature. For reuse, the Porapak Q columns were washed with 15 ml hexane and dried with a stream of nitrogen gas.

Tests of Attraction of Males to Airborne Pheromone Collections. Attraction of adult males to the crude pheromone extracts was tested in glass petri dishes (14 cm diam.). The Petri dishes were placed on white paper under diffuse overhead light at 19°–23°C. The samples of pheromone solution (10 μl equivalent to five females) to be tested were applied to a double disk (7 mm diam.) of filter paper. After 5 min, the disk was placed in the Petri dish.

In experiment 1 we compared the attraction of the males, in a choice test, to six pheromone samples collected at different times (viz. 4-hr collections) during the first 24 hr of operation in system A. The six pheromone-treated disks were placed at even intervals around the wall of the Petri dish. Eight to 20 adult males, which had emerged in the early morning, were introduced into the dish using a fine brush. They were observed for 45 or 60 min. The number of males displaying courtship or mating behavior on any of the disks was recorded. This behavior was characterized by a typical dance on and around the disk and by attempts to inseminate the filter paper. The test was repeated 12 times.

In experiment 2 we used the same set-up as in experiment 1, but with only four disks per Petri dish: one impregnated with the tested extract and three control disks. The six 4-hr Poropack collections, each obtained from three or five columns (from different days and systems), were tested with five replications. The tests were conducted from 0630 to 1130 hr, using 10 adult males for each replicate. A total of 1000 males were used for the entire experiment. During that time the number of males displaying courtship or mating behavior on the disks was recorded every 30 min (a total of 10 observations).

Olfactometer Bioassays. Pheromone bioassays were conducted, using a modified version of an olfactometer used by Schlyter and Lofquist (1985), to compare the attraction of adult males to virgin and mated females. The system consisted of an entry chamber, where the males were released, followed by a Y tube with either the airborne pheromone or clean air (control). Pheromone effluvia and air were drawn by a vacuum pump from the two chambers, one of them containing females. The males were allowed to advance to the point of division and to select the branch leading to the pheromone or control chamber. Females were verified as mated or virgin by the presence or absence, respectively, of ovisacs. The mated females had been fertilized 30 min prior to the experiment.

RESULTS

Emergence Patterns. The adult males started to emerge at 0200 hr, with a single peak of emergence recorded between 0300 and 0500 hr and the last individuals emerging around 1000 hr. On a single occasion, two males (0.14% of the total) emerged between 1900 and 2100 hr (Figure 1). Emergence of females was observed in various numbers at most hours of the day, with a maximum emergence occurring between 0500 and 0700 hr. The females usually did not emerge in the afternoon, and only small numbers were collected during the first half of the night (Figure 1); approximately 3% of all the females emerged between 1230 and 0300 hr.

The daily emergence periods were observed for the third-instar male lar-

vae, one in the early morning between 0300 and 0900 hr and the other in the evening between 1700 and 2100 hr (Figure 1).

Attraction to Airborne Pheromone Collections. As shown in Tables 1 and 2, no marked differences were recorded in the attraction of males to airborne pheromone extracts collected at different times. In experiment 1 (Table 1) the sample collected between 0430 and 0830 hr was the most attractive (16.7% of the males); about half the males did not respond to any of the collections (Table 1). In experment 2 (Table 2), males were almost equally attracted by all extracts, there being no marked differences between number of counts per treatment (number of times males displayed courtship or mating behavior with disks containing the sex pheromone from six collection periods). Only a small, but significant ($P < 0.05$) difference was found between the highest and lowest values, samples collected during 0030–0430 hr (22.8 counts), and 2030–0030 hr (15.7 counts), respectively.

Olfactometer Bioassay of Virgin vs. Mated Females. One single female, virgin or half an hour after mating, was sufficient to attract the male in the olfactometer system, whereas the control failed to attract any males (Table 3). In general, the attraction of males to virgin females was significantly ($P < 0.05$) greater than that of the mated females, but differences between the two groups were small. The relatively weak response in the tests of five virgin females and 15 mated females, 21% and 19%, respectively, was most probably due to the advanced age of the males in these tests (Table 3).

DISCUSSION

The emergence patterns of adult males and females of *M. josephi* are more or less synchronus. Although peak emergence of the males occurs 2 hr earlier than that of the females, the male may need this time to complete the emergence process and to locate host trees and females. This time gap may allow males to fly over long distances through the forest and reach other infested foci. Most females (97%) emerge during the time of the males' flight period. Thus, copulation is achieved on the emergence day, or at the latest, the next morning. Females of *M. feytaudi* display a similar emergence pattern, whereas male eclosion occurs between 1000 and 1600 hr (Riom and Fabre, 1977).

In the warm season, encounters between sexes occur during the early morning, when low temperature and high humidity enable the adult males of *M. josephi* to survive for many hours. From preliminary observations it seems that *M. josephi* males emerge and couple at temperatures above 9°C (Z. Mendel, unpublished). The low-temperature thresholds for flight of the male of the California red scale *Aonidiella aurantii* (Maskell) and the San Jose scale *Quadraspidiotus pernicious* (Comstock) (Homoptera: Diaspididae) are 15–18°C (Rice and Moreno, 1970) and 16°C (Rice and Hoyt, 1980), respectively. In these

TABLE 1. NUMBER OF MALES (% OF TOTAL IN PARENTHESIS) ATTRACTED TO FEMALE SEX PHEROMONE COLLECTED AT DIFFERENT HOURS OF THE DAY[a]

Time of the test (hr)	Number of tests	Total number of males	Time of pheromone collection (hours)						Males not responding to pheromone
			0030–0430	0430–0830	0830–1230	1230–1630	1630–2030	2030–0030	
0815–0900	6	64	4 (6.2)	1 (1.6)	1 (1.6)	7 (10.9)	8 (12.5)	12 (18.7)	31 (48.4)
0930–1030	3	57	2 (3.5)	19 (33.3)	2 (3.5)	5 (8.8)	5 (8.8)	2 (3.5)	22 (38.6)
1200–1245	3	32	0 (0)	5 (15.6)	0 (0)	1 (3.1)	0 (0)	7 (21.9)	19 (59.4)
Overall total[b]	12	153	6 (3.9)b	25 (16.7)a	3 (2.0)b	13 (8.5)ab	13 (8.5)ab	21 (13.7)a	72 (47.1)

[a]The experiment was conducted as a six-choice test in Petri dishes (for more details see text).
[b]Within the line means followed by the same letter are not significantly different (chi square test of k independent samples, Siegel, 1959).

TABLE 2. MEAN NUMBER OF MALES (COUNTS) ATTRACTED TO FEMALE SEX PHEROMONE COLLECTED IN DIFFERENT HOURS OF THE DAY[a]

Collection interval (hr)	Male attraction	
	Number of tests[b]	Mean number of male counts
0030–0430	15	22.8 a[c]
0430–0830	15	19.7 ab
0830–1230	25	20.3 ab
1230–1630	15	20.6 ab
1630–2030	15	16.9 ab
2030–0030	15	15.7 b

[a] Each test involved 10 males in a Petri dish with four disks: one impregnated with the test pheromone extract and three controls. The males did not respond to the control disks.
[b] The tests were conducted between 0630 and 1130 hr.
[c] Within column, means followed by the same letter are not significantly different ($P < 0.05$), Duncan's (1955) multiple range test.

TABLE 3. OLFACTOMETER ATTRACTION OF MALES TO VIRGIN AND MATED FEMALES OF *Matsucoccus josephi* VS. BLANK CONTROLS

Test object (no. of females)	Date	Hour	Number of males	Number (%) of males		
				Selecting female	Selecting the blank control	Failed to reach the dividing point (no response)
Virgin						
35	July 22	0945–1100	31	21 (68)	0	10 (32)
15	July 28	0915–1045	36	32 (89)	0	4 (11)
5	July 29	1345–1530	39	8 (21)	0	31 (79)
1	July 29	0700–0900	31	31 (100)	0	0
Combined (overall mean)				(67)[a]		
Mated						
15	July 28	1100–1200	42	8 (19)	0	34 (81)
5	July 29	0915–1115	22	16 (74)	1 (4)	5 (23)
1	July 30	0845–1015	33	17 (52)	0	16 (48)
Combined (overall mean)				(42)[a]		

[a] The overall treatment means (virgin vs. mated) were significantly different ($P < 0.05$) (determined by ANOVA; SAS Institute, 1982).

cases the onset of flight at a relatively high temperature may explain why most of the flight of males occurs during the afternoon (San Jose scale) or evening (California red scale) (Rice and Hoyt, 1980; Rice and Moreno, 1970). In another group of scale insects, the mealybugs (Homoptera: Pseudococcidae), in California and in Israel most males of *Planococcus citri* (Risso) were caught in pheromone traps between 0500 and 0800 hr, mainly during the first 2 hr after sunrise (Moreno et al., 1984; Tauber 1987). In Sardinia, two flight periods for males of *P. citri* were recorded: the main one in the early morning and the other one in the evening (Ortu and Delrio, 1982). The flight of the males of *Pseudococcus calceolariae* (Maskell) in Italy continues throughout the daylight hours with two clear peaks, the main one being around sunset (Rotundo and Tremblay, 1976). The males of *P. citri* fly at temperatures above 16°C, a threshold coinciding with that of the above diaspidids. McLaughlin and Ashley (1977) suggested that the emergence rhythm of the males of the white peach scale *Pseudaulacaspis pentagona* (Targioni-Tozzetti) is modified daily by the prevailing temperature cycle, apparently to ensure maximum survival and mating by the short-lived males.

Another factor that may affect the diurnal course of flight of the males is wind velocity. There are usually no strong air currents in the early morning that might confuse the flight of the males of *M. josephi* towards the pheromone sources. For example, males of the California red scale are unable to fly upwind to a pheromone source when air velocity exceeds 1 mph (Rice and Moreno, 1970); males of the San Jose scale achieve active flight at wind velocities of less than 4 mph (Rice and Hoyt, 1980). Males of *M. pini* (Green) usually couple on the lee side of the stem (Siewniak, 1976). Although the dimensions of the males of *Matsucoccus* are larger than those of diaspidids or pseudococcids, the former need to move relatively long distances to locate the females, whereas the latter two groups may find the first female at a distance of a few millimeters.

The third-instar male larvae of *M. josephi* migrate from the canopy and the stem during the same hours as the adults, and also in the late evening. Thus, there are two daily emergence periods, both under optimal temperature and humidity conditions.

Most insect species characteristically mate during a specific period of the day (Shorey, 1974). Among Lepidoptera, many species release their sex pheromone or respond to the attractant at a particular time of day and display a typical mating rhythm with seasonal shifts due to changes in temperature and photoperiod (Roelofs and Cardé, 1974; Shorey, 1974). However, some species such as the silkworm, *Bombyx mori* (L.), release the sex pheromone shortly after emergence until copulation or deposition of unfertilized eggs (Steinbrecht, 1964). Continuous release of sex pheromone is prevalent in scale insects. Rotundo and Tremblay (1980) investigated the sex behavior of *P. citri* and *P.*

calceolariae and found that pheromone release is uninterrupted until copulation takes place. In the present study, the behavior in *M. josephi* was similar. Release of sex pheromone throughout the day suggests that the females are ready to mate at all hours. They attract the males and are ready for insemination even before completing their emergence from the exuviae of the second-instar larvae (Z. Mendel, unpublished). Minor differences in pheromone release throughout the day may explain the minor preference of some pheromone collections when all samples were examined in the six-choice test (Table 1). In *M. josephi*, *P. citri*, and *P. calceolariae*, sex pheromone release does not coincide solely with flight of the males. Hence, we assume that the absence of a mechanism controlling the daily rate of pheromone release during the preinsemination period of females from two different families, Pseudococcidae and Margarodidae, may be an indication of a general phenomenon among members of the Coccoidea superfamily.

Reduction of sex pheromone release after mating, which is a general phenomenon in the Insecta, was noted by Tashiro and Moffitt (1968) in the California red scale. The female of this scale ceases to be attractive within 24 hr after mating, while virgin females remain attractive for 84 days. In *M. josephi* the female may attract males during 10 days after emergence (Bodenheimer and Neumark, 1955). We found that half an hour after mating, the female still attracts males, but to a lesser degree than virgins, suggesting reduced pheromone release.

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CHARACTERIZATION, SYNTHESIS, AND BEHAVIORAL RESPONSES TO SEX ATTRACTIVENESS PHEROMONES OF RED-SIDED GARTER SNAKES *(Thamnophis sirtalis parietalis)*

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Abstract—The sex attractiveness pheromone of the red-sided garter snake, *Thamnophis sirtalis parietalis*, has been characterized as a mixture of 13 long-chain (C_{29}–C_{37}) saturated and monounsaturated methyl ketones. Samples of the major unsaturated ketones in the mixture, (*Z*)-24-tritriaconten-2-one **(7)** and (*Z*)-26-pentatriaconten-2-one **(10)**, and their saturated analogs, 2-tritriacontanone **(8)** and 2-pentatriacontanone **(11)** were prepared by unambiguous synthesis. In field bioassay tests, male garter snakes were presented with natural and synthetic pheromone components both individually and as a mixture. Males exhibited courtship behavior to the synthetic compounds approximating the natural blend.

Key Words—Pheromones, methyl ketones, red-sided garter snake, *Thamnophis sirtalis parietalis*, sexual behavior, bioassay, Reptilia.

INTRODUCTION

The study of chemical messages or semiochemicals in vertebrates is difficult because animals rarely rely on a single sensory input. For instance, in nature and even in the laboratory many mammals do not rely exclusively on olfaction for the initiation or maintenance of normal sex behavior (Singer et al., 1976). In most vertebrates the odors functioning in recognition of species, group, individual, or physiological state may consist of dozens of compounds (Duvall, 1986). More confounding is that positive responses in bioassays frequently disappear before fractionation procedures reach the level of single compounds (Müller-Schwarze et al., 1986).

The chemical communication system of garter snakes (*Thamnophis*) is an ideal model for the study of vertebrate semiochemicals. The garter snake possesses a relatively simple suite of semiochemicals, and the behaviors measured in the bioassay are robust, simple to quantify, and unequivocal in that they are only observed in a reproductive context in response to pheromonal cues. In addition, the sensory mechanisms involved in reception and perception of chemical cues in garter snakes are confined to one well-understood system. Noble (1937) purported to show that both the olfactory and the vomeronasal system mediate the reception and response to pheromones in the garter snake. He demonstrated that certain chemical manipulations of the pheromone on the female's back could abolish courtship by sexually active males. He also devised a number of ingenious experiments to determine which sensory modality the males were using to recognize and court females. Auditory, visual, and tactile cues were eliminated, leading him to conclude that the tongue delivers chemicals to the vomeronasal organ in the roof of the mouth. More recent work by Kubie *et al.* (1978) demonstrated unequivocally that the vomeronasal organ is the only means by which the skin-derived pheromone cues can be perceived.

Unlike the olfactory system, the vomeronasal system is extremely sensitive to nonvolatile chemical stimuli. Sheffield et al. (1968) demonstrated that newborn garter snakes detect with their vomeronasal organs a component of earthworm washes of molecular weight greater than 5000 daltons. More recently, Wang et al. (1988) isolated an approximately 20,000-dalton protein from aqueous earthworm extracts which is a chemoattractant for garter snakes. Recent investigations by Burghardt et al. (1988) also have isolated components in earthworms and minnows that elicit feeding behavior from garter snakes. Chloroform–methanol extracts of earthworms yielded a high-molecular-weight (432,000) component as well as a previously unknown low-molecular-weight component between 1350 and 1800 daltons.

The red-sided garter snake (*Thamnophis sirtalis parietalis*) is the most northerly living reptile in North America (Logier and Toner, 1961). As such, it is constrained to hibernate up to eight months of the year in underground dens

or hibernacula. In Manitoba, Canada, males emerge in late April and several thousand aggregate at the den entrance. Over the next three to four weeks, females emerge singly or in small groups. Males search for sexually attractive females and are initially attracted to movement (Joy and Crews, 1988), but they must actually come into contact with a female before courtship begins.

Upon encountering an unmated female, the male presses his chin onto her dorsal skin and emits very short, rapid tongue flicks. At the same time he traverses up and down the female, turning back at her head and tail, coming to rest with his head resting behind hers. Once stationary, the male's body undulates in caudocephalic waves. Both chin-rubbing and caudocephalic waves are observed only during mating and are not associated with any other behaviors. Many males will court a female simultaneously, forming a "mating ball" of 10–100 males. However, only one male will mate with the female while the others disperse to seek other unmated females.

Similar studies by Andrén and his colleagues have shown that in other snakes reproductive behaviors also are elicited in response to skin-derived pheromones (Andrén, 1982, 1986). Courtship in the European adder, *Vipera berus*, occurs when males begin chin-rubbing and tongue-flicking the dorsal surfaces of females. He has demonstrated that pheromone cues are gathered by direct contact with the tongue and are sensed by means of the vomeronasal organ.

It is important to note that the sex attractiveness pheromone produced by female garter snakes serves to advertise the female's unmated status. It does not imply attraction of males from a distance in that the pheromone is known to be a contact pheromone. Many snakes are known to trail conspecifics and garter snakes are especially adept at following trails. Indeed, male garter snakes will preferentially trail females during the breeding season, presumably basing this behavior on the ability to follow cuticular cues left on the substrate (Noble, 1937; Ford, 1978, 1981). Whether these trailing pheromones are the same as the sex attractiveness pheromone remains to be seen.

In a previous report (Mason et al., 1989), we isolated a series of long-chain methyl ketones that serve as the sex attractiveness pheromones in this species. In addition, we described the initial characterization and synthesis of two saturated methyl ketones. In the current study, we present the complete characterization of the natural material as well as the synthesis and field testing of a synthetic mixture of both saturated and unsaturated methyl ketones.

METHODS AND MATERIALS

Chemical Analyses. Gas chromatographic analyses were performed on a Shimadzu GC-12A equipped with a 30-m × 0.53-mm-ID column with a bonded DB-17 phase. [^{1}H]- and [^{13}C]NMR spectra were obtained with a Varian XL-

200 spectrometer from $CDCl_3$ solutions. Mass spectra were obtained using an LKB-9000 GC-MS fitted with a 30-m × 0.53-mm-ID column with a bonded DB-17 phase, or a Finnigan model 4500 GC-MS fitted with a 30-m × 0.32-mm-ID column with a bonded DB-1 phase. High-resolution mass spectra were obtained using a VG-7070F in the EI mode at an ionizing voltage of 70 eV. All boiling points and melting points are uncorrected.

Derivatization. A small sample (ca. 10 mg) of the natural methyl ketone fraction (Mason et al., 1989) was treated with dimethyldisulfide and a catalytic amount of iodine and after 12 hr, worked up in the usual manner (Buser et al., 1983). The resulting mixture contained bisthiomethyl derivatives of the unsaturated ketones, **4, 7, 9, 10, 12,** and **13,** whose mass spectra had characteristic ions at m/z = 58 and 61, as well as the following pairs of intense ions: **4** + $(CH_3S)_2$: m/z = 173, 369; **7** + $(CH_3S)_2$: m/z = 173, 397; **9** + $(CH_3S)_2$: m/z = 173, 411; **10** + $(CH_2S)_2$: m/z = 173, 425; **12** + $(CH_2S)_2$: m/z = 173, 439; **13** + $(CH_2S)_2$: m/z = 173, 453.

This mixture was taken up in pyridine (0.1 ml), treated with 10 mg of O-methylhydroxylamine hydrochloride, and heated to 100°C for 15 min. Analysis of the resulting mixture by GC-MS (oven programmed 180–320°C) revealed the presence of three late-eluting components in a 1:12:15 ratio with mass spectra indicative of the bisthiomethyl-O-methoxime derivatives of **4, 7,** and **10,** respectively: MS m/z relative intensity) peak 1: 571(0.5, M+), 398(25), 174(10), 173(60), 100(25), 97(55), 95(30), 87(100), 83(60), 81(35), 71(30), 70(30), 69(65), 61(30), 57(90), 55(70), 43(65), 41(40). Peak 2: 599(10, M+), 552(7), 427(20), 426(61), 173(100), 172(27), 100(20), 97(36), 95(33), 87(96), 83(70), 81(36), 71(25), 70(30), 69(80), 61(43), 57(71), 55(85), 43(59), 41(35). Peak 3: 627(5, M+), 580(3), 455(7), 454(45), 173(100), 172(20), 100(15), 97(35), 95(30), 87(75), 83(65), 81(25), 70(25), 71(30), 69(80), 61(45), 57(75), 55(70), 43(45), 41(30).

*Synthesis of 2-(13-Hydroxytridecyl)-2-methyldioxolane (**14,** N = 12).* Ethyl acetoacetate (30 ml, 0.23 mol) was added to a rapidly stirred ethanolic solution of sodium ethoxide made of dissolving 5.0 g of sodium in 200 ml of absolute ethanol. After 0.5 hr, a solution containing 58.3 g (0.22 mol) of 12-bromododecanol (Kang *et al.*, 1985) in 50 ml of ethanol was added dropwise, and the mixture was heated to reflux under anhydrous conditions overnight. The mixture was cooled, neutralized with a few drops of glacial acetic acid, and the solvent was removed under reduced pressure. The residue was taken up in ether, washed with water, and after removal of the solvent, the residue from the ether solution was stirred overnight with 150 ml of aqueous 10% NaOH. This mixture was carefully acidified with 50% H_2SO_4 and heated in a warm water bath until gas evolution ceased. Upon cooling, the mixture was extracted with ether, and after removal of the solvent, the residue from the ether solution was taken up in benzene, combined with 20 ml of ethylene glycol and 0.2 g of *p*-toluene-

sulfonic acid, and heated to reflux under a Dean-Stark trap for 4 hr until the rapid evolution of water ceased. The mixture was cooled, washed with saturated $NaHCO_3$, dried over anhydrous $MgSO_4$, filtered and distilled. Kugelrohr distillation provided 28.45 g (49% yield) of hydroxyketal **14** (N = 12), bp 160–170°C (0.35 mm Hg); [^{1}H]NMR δ = 3.9(4H, m), 3.6(2H, t, J = 6Hz), 2.1(1H, br s), 1.65–1.45(4H, m), 1.28(3H, s), 1.25(2OH, m); [^{13}C]NMR δ = 110.22, 64.53(2C), 62.96, 39.19, 32.74, 29.83–29.13(8C), 25.70, 24.05, 23.85; MS *m/z* (relative intensity) 271(9, M^+-15), 99(5), 88(5), 87(100), 69(5), 55(15), 43(30), 41(10); HRMS *m/z* 271.2276 ($C_{16}H_{31}O_3$, M^+-15, calcd. 271.2273).

Synthesis of (Z)-*24-Tritriaconten-2-one* ***(7)***. A well-stirred solution containing 2.0 g (7 mmol) of hydroxyketal **14** and 1 ml of pyridine in 10 ml of chloroform was cooled to 0°C and treated with 0.8 g of methanesulfonyl chloride. The resulting mixture was stirred for 1 hr at 0°C, and then treated with 30 ml of ether and 30 ml of saturated $NaHCO_3$ solution. The ether solution was separated, dried over anhydrous $MgSO_4$, filtered, and the solvent removed in vacuo to 0.1 mm Hg. The fluffy white residue was taken up in anhydrous THF, filtered, and the solvent was removed in vacuo to 0.1 mm Hg to provide the unstable methanesulfonate ester **15** (N = 12); MS *m/z* (relative intensity) 349(0.5, M^+-15), 253(5), 99(8), 88(9), 87(100), 55(15), 43(20), 41(10). This material was taken up in 20 ml of anhydrous THF.

A solution of oleylmagnesium bromide, decanted from the reaction of 3 g of oleyl bromide with 1.0 g of magnesium in 50 ml of anhydrous THF, was cooled to −78°C under a nitrogen atmosphere and treated with the above THF solution of **15** (N = 12). After the addition of 4 ml of a freshly prepared 0.2 M solution of Li_2CuCl_4 in THF, the resulting blue–purple mixture was maintained at 0°C overnight. Following the addition of 3 ml of water, the mixture was stirred for 0.5 hr and the organic layer was decanted from the solids, cooled in an ice bath, and treated with 10 ml of water and 3 ml of 70% $HClO_4$. After 3 hr, the mixture was carefully neutralized with solid $NaHCO_3$, extracted with ether, and the ether solution was dried over anhydrous $MgSO_4$. After filtration, the solvent was removed in vacuo and the residue was taken up in 20 ml of hot hexane, allowed to cool, and filtered again. The solvent was removed from the resulting mixture, and the residue was purified by chromatography over 200 g of activity III alumina (50 : 1 hexane–ether) to provide 1.32 g (40% yield) of **7**: mp 52–53°C; [^{1}H]NMR δ = 5.35(2H, br t), 2.41(2H, t, J = 7 Hz), 2.17(3H, s), 2.04(4H, m), 1.65–1.2(50H, m), 0.88(3H, br t); [^{13}C]NMR δ = 209.48, 130.13(2C), 44.06, 32.14, 30.01–29.55(22C), 27.45, 24.13, 22.91, 14.32; MS *m/z* (relative intensity) 476(6,M+), 461(1), 459(3), 458(12), 418(2), 125(25), 111(20), 109(18), 97(40), 96(33), 95(28), 85(11), 83(50), 82(30), 81(27), 71(60), 69(55), 59(35), 58(43), 57(44), 55(70), 43(100), 41(25); HRMS *m/z* 476.4944 ($C_{33}H_{64}O$, M^+ calcd. 476.4957).

Synthesis of 2-Tritriacontanone **(8)**. A solution containing 0.5 g (1.05

mmol) of **7** in 50 ml of hexane was hydrogenated over 47 mg of 5% palladium on carbon at 3 atm of pressure for 1 hr. Filtration of the resulting mixture through a short pad of Florisil and removal of the solvent in vacuo provide 0.47 g (94% yield) of ketone **8:** mp 79–80°C; MS *m/z* (relative intensity) 478(20, M^+), 463(4), 460(6), 420(1), 97(22), 96(18), 95(12), 85(27), 83(25), 82(20), 81(11), 71(65), 69(28), 59(100), 58(70), 57(55), 55(40), 43(95), 41(28); HRMS *m/z* 478.5099 ($C_{33}H_{66}O$, M^+ calcd. 478.5113).

Synthesis of 2-(15-Hydroxypentadecyl)-2-methyldioxolane ***16*** *(N = 14).* 14-Bromotetradecanol, 18.8 g (64 mmol) was carried through the steps described above for **14** (N = 12), to provide, after Kugelrohr distillation 8.6 g (43% yield) of **16:** bp 210–220°C (0.1 mm Hg); [^{1}H]NMR δ = 3.92(4H, m), 3.62(2H, t, J = 7 Hz), 1.9(1H, br s), 1.7–1.5(4H, m), 1.3(3H, s), 1.26(24H, m); [^{13}C]NMR δ = 110.16, 64.49(2C), 62.82, 39.16, 32.74, 29.53(10C), 25.71, 24.02, 23.61; MS *m/z* (relative intensity) 299(9, M^+-15), 281(1), 99(3), 88(5), 87(100), 55(10), 43(20), 41(7); HRMS *m/z* 299.2581 ($C_{18}H_{35}O_3$, M^+-15 calcd. 299.2586).

Synthesis of (Z)*-26-Pentatriaconten-2-one* ***(10).*** The hydroxy ketal **16,** 2.1 g (6.7 mmol), was converted to its unstable methanesulfonate ester **17** (N = 14) in the manner described above: MS *m/z* (relative intensity) 377(4, M^+-15), 281(5), 99(3), 88(6), 87(100), 55(10), 43(18), 41(7). In the manner described above, this material was allowed to react with an excess of oleylmagnesium bromide in the presence of lithium tetrachlorocuprate to provide, after chromatography over activity III alumina, 1.26 g (39% yield) of **10:** mp 56–57°C; [^{1}H]NMR δ = 5.45(2H, br t), 2.43(2H, t, J = 7 Hz), 2.17(3H, s), 2.04(4H, m), 1.65–1.2(54H, m), 0.92(3H, br t); [^{13}C]NMR δ = 209.49, 130.14(2C), 44.07, 32.15, 30.36–29.65(24C), 27.45, 24.14, 22.91, 14.32; MS *m/z* (relative intensity) 504(10, M^+), 489(2), 486(18), 446(3), 125(25), 111(2), 109(18), 97(44), 96(35), 95(30), 83(55), 82(35), 81(27), 71(60), 69(55), 67(25), 59(38), 58(45), 57(50), 55(70), 43(100), 41(25); HRMS *m/z* 504.5275 ($C_{35}H_{68}O$, M^+, calcd. 504.5270).

Synthesis of 2-Pentatriacontanone **(11).** In the manner described above, a hexane solution of **10** was hydrogenated to provide 0.46 g (92% yield) of **11:** mp 80–82°C; MS *m/z* (relative intensity) 506(10, M^+), 491(1), 488(5), 448(1), 97(3), 96(20), 95(25), 85(33), 83(40), 82(25), 81(28), 71(95), 69(50), 59(75), 58(65), 57(70), 55(62), 43(100), 41(45); HRMS *m/z* 506.5428 ($C_{35}H_{70}O$, M^+ calcd. 506.5427).

Synthetic Mixtures for Bioassay. The average snake from these populations is 60 cm long and 3 cm in diameter (Gregory, 1974), thus the average dorsal surface area is 282 cm^2. Chromatography over Al_2O_3 of hexane extracted lipids from 18 snakes provided 117 mg of ketones or 6.5 mg/snake. Analysis by GC-MS revealed that 88.4% of the sample consists of compounds **2, 5, 7, 8, 10, 11,** in a ratio of 4:7.5:6:6:5:1. Thus, one female snake has 0.67 mg **2,** 1.25

mg **5**, 1.0 mg **8**, 0.8 mg **7**, 1.0 mg **10**, and 0.16 mg **11**. Based on the synthetic material available, a mixture consisting of 70 mg **2**, 40 mg **5**, 100 mg **8**, 80 mg **7**, 100 mg **10**, and 20 mg **11** [100 female equivalents (FE)] in hexane was utilized as a stock solution for the bioassays. The major difference from the natural mixture is the smaller amount of the major saturated component **(5)** in the synthetic mixture due to the lack of synthetic **5** available, and the necessity of having a sufficient quantity of the mixtures for the bioassays.

A mixture approximating the remaining skin lipids was made up of 41% oleic acid, 16% palmitic acid, 20% cholesterol, and 20% tripalmitin in hexane. Using 38 mg as the average amount of total lipids per snake (Mason et al., 1987), 31.5 mg of this mixture is 1 FE of remaining skin lipids. This mixture, the mixture of ketones, and the individual ketones **7, 8,** and **10,** were dissolved in hexane and diluted in terms of female equivalents as shown in Tables 2 and 3 below.

Field Bioassay. All behavioral tests were conducted in the field at Narcisse Community Pasture, Manitoba, Canada, during the spring emergence of the garter snakes in May 1989. Testing of individual saturated and unsaturated ketones **7, 8,** and **10** was conducted with the same bioassay procedure used in previous studies (Mason and Crews, 1985; Mason et al., 1989) to facilitate direct comparisons of data. In these tests, varying concentrations (0.01–10 FE/ml) of individual synthetic methyl ketones in hexane were applied to an area 16 × 16 cm on paper towels. After applying the solutions to the paper towels, the paper towels were placed in the den where courting males had ready access to them. Untreated and hexane-soaked paper towels were used as controls. Males responding with tongue-flicking and chin-rubbing for 10 sec or more were scored as positive responders. Three replicate tests were conducted for each compound.

In order to test for possible synergistic effects of ketones working as a mixture as found naturally, the synthetic ketones available were tested together in proportion to their relative abundance in the snake skin. All ketones were tested both alone and with the lipid.

As for the individual ketones above, the synthetic mixture of ketones was tested at varying concentrations (0.001–1 FE/ml). Three behaviors were monitored and scored in response to the synthetic pheromone mix. Duration of courtship was the number of seconds courting males exhibited courtship behavior in response to the test compound. Total tongue-flicks elicited by a given mixture were counted for the duration of a 1-min test. Finally, the tongue-flick rate was recorded as tongue-flicks per minute. For each concentration of the synthetic pheromone mix a total of 10 different males were observed.

Statistics. Physical parameters of male courtship response to each experimental group were compared by one-way analysis of variance ANOVA (SAS Institute Inc., 1985). Responses to ketone mixes both with and without lipids

were analyzed by two-way ANOVA with concentration of ketones and presence or absence of lipid as factors. Unplanned comparisons between means were identified by Duncan's multiple-range test. A simple linear regression analysis was conducted on the courtship data to determine the predictability of courtship versus concentration of the synthetic pheromone mix. Courtship responses were normalized by log transformation before statistical analysis. Alpha was set at 0.05 for all statistical tests.

RESULTS

The skin lipids of female *Thamnophis sirtalis parietalis* were extracted and fractionated as previously described (Mason et al., 1989). Initial spectroscopic examination of the bioactive fraction revealed the presence of methyl ketones (IR 1712 cm^{-1}; [^{1}H]NMR, δ = 2.10, singlet, CH_3CO and 2.38, triplet, CH_2COCH_3) along with unconjugated *Z* double bonds (IR 718 cm^{-1}; [^{1}H]NMR δ = 5.32) (Mason et al., 1989).

Complete GC-MS analysis of the ketone fraction revealed the presence of 13 methyl ketones (**1-13,** Table 1). Seven of these [2-heptacosanone (**1**), 2-nonacosanone (**2**), 2-triacontanone (**3**), 2-hentriacontanone (**5**), 2-dotriacontanone (**6**), 2-tritriacontanone (**8**), 2-pentatriacontanone (**11**)] had mass spectra in accord with saturated methyl ketones, showing a molecular ion, and losses of 15, 18, and 60 as well as an intense ion at *m/z* = 58 and a base peak at *m/z* = 59 (Sharkey et al, 1956). The molecular ions and fragmentation patterns of the remaining ketones indicated monounsaturated methyl ketones. To determine the double-bond position, the mixture was subjected to bisthiomethylation (Buser et al., 1983). The mass spectra of the derivatives showed two abundant ions resulting from cleavage between vicinal thiomethyl groups. One of these ions in each derivative, *m/z* = 173, indicated the double bond to be the same distance (ω-9) from the end of the molecule distal to the ketone. To establish the relationship between the carbonyl and olefinic functions, the derivatized mixture was treated with O-methylhydroxylamine hydrochloride. Subsequent GC-MS analysis provided readily discernible mass spectra for the bisthiomethyl-O-methyloxime derivatives of **4, 7,** and **10.** Figure 1 depicts the mass spectral fragmentation pattern of the bisthiomethyl methoxime derivative of **7**. Fragments at *m/z* = 87 and 100 are the McLafferty rearrangement and α-cleavage ions characteristic of methyl ketone O-methyloximes (Budzikiewicz et al., 1967), and the ions at *m/z* = 426 and 173 result from cleavage between the vicinal thiomethyl groups. Since the nitrogen of the methoxime is incorporated in the larger, even-mass fragment ion, **7** is (*Z*)-24-tritriaconten-2-one. The unchanged ion at *m/z* = 173 common to all the unsaturated ketone dimethyl disulfide derivatives permits the assigment of **4** as (*Z*)-22-hentriaconten-2-one,

TABLE 1. GC-MS ANALYSIS OF METHYL KETONES FROM FEMALE *T. sirtalis parietalis*

Compound	R_t (min)	Relative %	MS: *m/z* (relative intensity) Characteristic Ions
1	16:29	1.24	394(25, M+), 379(6), 376(14), 334(15), 109(5), 97(11), 96(10), 85(10), 71(45), 59(100), 58(60), 57(30)
2	17:49	11.4	422(55, M+), 407(13), 404(20), 362(11), 96(30), 85(25), 71(60), 59(100), 58(80), 57(45), 55(30)
3	18:45	0.74	436(10, M+), 421(3), 418(4), 376(2), 96(23), 85(23), 71(50), 59(100), 58(75), 57(40), 55(27)
4	18:55	1.24	448(6, M+), 433(2), 430(12), 125(2), 111(30), 109(45), 97(63), 96(56), 83(78), 71(80), 69(77), 59(60), 58(60), 57(73), 55(100)
5	19:08	22.8	450(8, M+), 435(3), 432(3), 390(5), 96(15), 85(11), 83(15), 71(40), 59(100), 58(75), 57(45), 55(30)
6	19:45	1.48	464(20, M+), 449(9), 446(11), 85(50), 71(55), 59(65), 58(100), 57(50), 55(55)
7	20:22	14.4	476(7, M+), 458(16), 418(5), 125(23), 123(17), 111(24), 109(35), 97(67), 96(55), 95(57), 83(84), 71(70), 69(90), 59(60), 58(70), 57(65), 55(100)
8	20:32	17.9	478(35, M+), 463(11), 460(25), 418(30), 96(11), 83(11), 71(40), 59(100), 58(54), 57(25), 55(15)
9	21:03	2.72	490(5, M+), 475(6), 472(12), 432(4), 125(16), 123(17), 109(41), 97(75), 83(94), 69(95), 59(62), 58(74), 57(75), 55(100)
10	22:00	18.9	504(8, M+), 489(3), 486(17), 446(5), 125(24), 123(18), 111(28), 109(30), 97(67), 96(55), 95(50), 83(86), 71(70), 69(86), 59(57), 58(64), 57(68), 55(100)
11	22:04	2.48	506(5, M+), 488(7), 446(3), 109(20), 97(35), 83(57), 71(57), 59(100), 58(68), 57(65), 55(58)
12	22:32	1.48	518(7, M+), 500(9), 458(3), 125(17), 109(32), 97(66), 83(77), 69(80), 59(63), 58(64), 57(75), 55(100)
13	23:26	3.22	532(6, M+), 514(13), 474(4), 125(22), 109(30), 97(70), 83(96), 69(92), 59(68), 58(64), 57(70), 55(100)

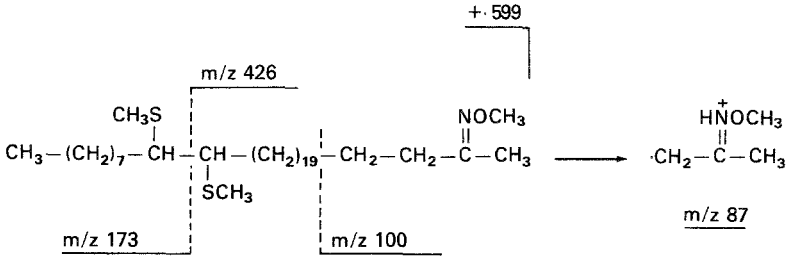


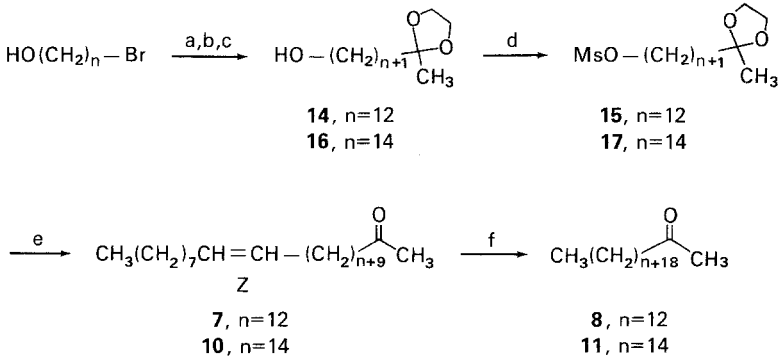
FIG. 1. Suggested interpretation of the MS of the bisthiomethyl-*O*-methyloxime derivative of **7**.

9 as (*Z*)-25-tetratriaconten-2-one, **10** as (*Z*)-26-pentatriaconten-2-one, **12** as (*Z*)-27-hexatriaconten-2-one, and **13** as (*Z*)-28-heptatriaconten-2-one.

Since **7** and **10** are the major unsaturated ketones in the mixture, their synthesis was undertaken to provide them as well as their saturated analogs **8** and **11** for subsequent bioassays.

The preparations of **7** and **10** were based on the copper-catalyzed coupling of the Grignard reagent prepared from oleyl bromide (Kocienski et al., 1977) with the appropriate methanesulfonate (Scheme 1). Ethyl acetoacetate was condensed with 12-bromododecanol (Kang et al., 1985), to provide, after routine saponification, decarboxylation, and ketalization, the hydroxy ketal **14** in moderate yield. The unstable methanesulfonate ester **15** was prepared in the usual manner and carefully dried. The condensation of this methanesulfonate with oleylmagnesium bromide in the presence of lithium tetrachlorocuprate (Fouquet and Schlosser, 1974) furnished the unsaturated ketone **7,** which could be isolated in gram quantities by simple chromatography over activity III alumina. In the same way, **10** was prepared from 14-bromotetradecanol (Valicenti et al., 1985). Samples of the saturated ketones **8** and **11** were obtained by hydrogenation of **7** and **10**, respectively, over a 5% Pd/C catalyst.

Behavior. Behavioral responses of sexually active male garter snakes to individual synthetic methyl ketones **7, 8,** and **10** are shown in Table 2. It appears that males will respond to unsaturated methyl ketones at lower concentrations than required to elicit courtship from individual saturated ketones. These results follow the general trend demonstrated in an earlier study with isolated natural saturated and unsaturated methyl ketones (Mason et al., 1989). Males did not respond to the ketones at all at concentrations less than 1 FE, indicating that the concentrations of ketones on females are near the threshold of the males' ability to detect them. Interestingly, partially dried hexane controls, as com-



SCHEME 1. (a) $CH_3COCH_2CO_2Et$, EtONa; (b) ^-OH, H^+; (c) $(CH_2OH)_2$, ptsa; (d) MsCl, Py; (e) oleylMgBr, Li_2CuCl_4; (f) Pd/C, H_2.

TABLE 2. BEHAVIORAL RESPONSES TO INDIVIDUAL SYNTHETIC METHYL KETONES[a]

	Solvent blank	Concentration (FE/ml) 0.01	0.1	1	2	10
C_{33}	0[b]	0	0	0	1.3 ± 0.88	3.67 ± 0.33
$C_{33:1}$	0	0	0	0.33 ± 0.33	3 ± 1.15	2 ± 0
$C_{35:1}$	0	0	0	1.33 ± 0.88	3.3 ± 1.76	2.67 ± 1.2

[a] Tests conducted at Narcisse Community Pasture, Manitoba, Canada, May 10–12, 1989. Results of triplicate test. Testing was monitored for 1 hr/day. Those males that responded with increased tongue-flicking and chin rubbing for 10 sec or more were given a positive score.
[b] Number of males responding with courtship (mean ± SE).

pared to untreated paper towels, proved very aversive to males. Thus, not only did males respond positively to the ketones, but they also overcame a strong aversion to the hexane in which the ketones were dissolved. Males never demonstrated any interest in the lipid controls.

Results of courtship tests with the synthetic ketone mixture alone were compared to the same mixture including the supplemental lipids. A two-way ANOVA for the duration of courtship data indicated that ketones plus lipids elicited significantly more courtship than ketones alone [$F(5,94) = 6.25$, $P < 0.001$]. Similarly, the total number of tongue-flicks elicited also differed significantly [$F(5,94) = 27.40$, $P < 0.001$]. Thus, we conclude that the additional lipids may be serving as a matrix that makes the long-chain methyl ketones more readily available for males. Because these ketones are a contact pheromone, the lipids may be facilitating the physical transfer of chemical cues from the female's back to the male's vomeronasal organ.

The responses of males to the synthetic ketone mixture plus lipids are listed in Table 3. In each case, the one-way ANOVAs for duration of courtship, total tongue-flicks, and tongue-flick rate were significantly different between concentrations. However, these ANOVAs included the two controls of solvent and lipids, which never elicited any courtship. Likewise, the natural material was almost always 5–10 times greater in level of response. Thus, an ANOVA was calculated on just the five different concentrations of synthetic mixtures. The duration of courtship was found to be significantly different between concentrations [$F(4,45) = 12.12$, $P < 0.001$] while total tongue-flicks were also significantly different [$F(4,45) = 11.47$, $P < 0.001$]. However, when the natural material and controls are factored out of the tongue-flick frequency data, there are no significant differences between the five concentrations [$F(4,45) = 2.46$, $P < 0.05$]. Thus, it appears that although males will respond differentially with tongue-flicks and duration of courtship response, they do not alter their tongue-flick rate as the concentration of material changes.

TABLE 3. SCORES (DURATION, NUMBER, OR FREQUENCY) OF MALE COURTSHIP RESPONSES TO SYNTHETIC PHEROMONE MIX AND CONTROLS[a]

	FE/ml (mean ± SE, $N = 10$)								
	Hexane	Lipids	Natural (1 FE)	1	0.5	0.1	0.01	0.001	ANOVA
Duration of courtship (sec)	0a	0a	110.6 ± 16.4b	20.1 ± 3.84c	8.7 ± 1.3c	6.2 ± 1.02c	4.9 ± 0.48c	3.8 ± 0.53c	$F(7, 72) = 39.06$, $P < 0.001$
Total tongue-flicks elicited	0a	0a	136.8 ± 4.65b	27.8 ± 5.97c	12.6 ± 1.45c,d	8.5 ± 1.37d	6.1 ± 0.89d	3.3 ± 0.4d	$F(7, 72) = 267.9$, $P < 0.001$
Tongue-flick frequency (tongue-flicks/min)	0a	0a	142.1 ± 2.52b	107.8 ± 11.51b,c	83.4 ± 9.53c	75.9 ± 10.24c	70.5 ± 9.22c	70.4 ± 8.91	$F(7, 72) = 38.1$, $P < 0.001$

[a] Letters common in a row denote mean values that do not differ significantly (Duncan's multiple-range test).

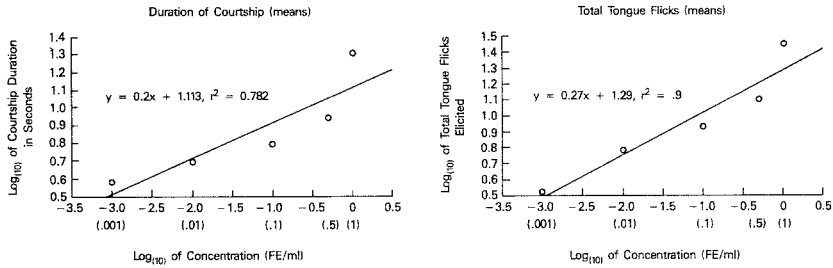


FIG. 2 Responses of courting male *Thamnophis sirtalis parietalis* towards five different concentrations of synthetic pheromone mix. Values represent the duration of courtship in seconds (left panel) and the total number of tongue-flicks elicited towards the synthetic mix (right panel). Each datum represents the mean of 10 observations.

Finally, we plotted the five concentrations of ketones plus lipid mixture as the log of concentration versus the log of response for the duration of courtship data and total tongue-flicks elicited and performed a simple linear regression analysis. The results are shown in Figure 2. The straight line fitted to the data in both cases was significant accounting for 78% of the variance in the former and 90% of the variance in the latter.

DISCUSSION

Beginning with studies by Noble and his colleagues in the 1930s, there are nearly 50 years of data documenting, in squamate reptiles, specialized behaviors of touching the conspecifics' skin with the tongue and then placing the tongue tip close to, if not in, the buccal opening of the Jacobson's or vomeronasal organ (Maderson, 1986). Graves et al. (1986) and Maderson (1986) have proposed that the integument of all extant amniotes, and probably that of their ancestors, is or was to varying degrees an uncontrolled semiochemical source.

All higher vertebrates possess corneous tissues that contain intra- and extracellular lipids that affect the waterproofing of the integument. These lipids have been shown to impede transcutaneous water loss in several species of squamate reptiles (Roberts and Lillywhite, 1980, 1983; Lillywhite and Maderson, 1982; Baeyens and Rountree, 1983; Burken et al., 1985a). Indeed, many investigators have identified lipids in the skin secretions of snakes (Jackson and Sharaway, 1978; Tsumita et al., 1979; Birkby et al., 1982; Burken et al., 1985b; Mason et al., 1987). Principle among these are studies by Ahern and Downing (1974) and Schell and Weldon (1985), who first identified long-chain methyl ketones in the skin of snakes.

There is now a growing body of evidence that strongly suggests that the lipid molecules within or beneath epidermal tissues "seep" through to the skin

surface (Garstka and Crews, 1981; Garstka et al., 1982; Maderson, 1986). In birds, lipids are excreted from epidermal cells undergoing keratinization (Menon et al., 1981). These lipids then appear to be extruded to the skin surface through pores (Purton, 1986). Similar pores have been described on the dorsal surface of snakes (Stille, 1987; Chiasson and Lowe, 1989; Chiasson et al., 1989). Chiasson et al. (1989) suggest that these pores in reptiles serve as the surface opening by which lipids produced in the epidermis reach the skin surface. In this model, the lipid semiochemicals would be derived from those lipids that serve as the integumental water barrier. Thus, the integumental lipids would have been protoadapted (Gans, 1979) or exapted (Gould and Vrba, 1982) to serve an additional role, that of semiochemical communication.

Although females may engage in deep breathing during courtship, which may aid in expressing the skin pheromone, the "passive" secretion of semiochemicals in snakes is supported by numerous field observations of courtship in garter snakes. The response to the female attractiveness pheromone by courting males is very robust. Males are not uncommonly observed courting and even mating with females that have been dead for several days (Aleksiuk and Gregory, 1974; Garstka et al., 1982). Thus, the female does not need to actively secrete the attractiveness pheromone, and it seems to remain intact on her skin surface unaltered for several days. These observations demonstrate not only the powerful and long-term effect of these skin lipids as pheromones, but also point out the fact that, in garter snakes, the female's behavior is not essential for eliciting male sexual behavior.

The fact that skin surface lipids are serving as semiochemicals merits comment. Both the number of different products and their relatively high molecular weight may, after cursory examination, argue against their usefulness as species-specific semiochemicals. In the early years of insect semiochemical research, a pheromone was usually reported as a single compound. The concept of "one insect–one specific compound" became strongly implanted among researchers in the developing pheromone field (Hadley, 1985). Field trials on these single compounds often proved to elicit a weaker response than a multicomponent mixture. Moreover, the purer the compound, the more disappointing the results (Silverstein, 1977). Our results show a similar trend.

The first definitive demonstration of a multicomponent attractant pheromone was provided by Silverstein et al. (1966), who showed that the pheromone produced by the male bark beetle, *Ips paraconfusus*, which attracts both males and females, was a mixture of three terpene alcohols, none of which was particularly active by itself in the field. Subsequent studies in widely ranging taxa have shown that, although there are pheromones that consist of a single active compound, multicomponent systems or pheromone blends predominate. Indeed, most vertebrate odors functioning in recognition of species, group, individual, or physiological state consist of dozens of compounds (Duvall, 1986).

Another idea that has become entrenched over the years is that all pheromones must be somewhat volatile. Bossert and Wilson (1963) proposed a set

of theoretical constraints on the chemical properties of pheromones. At this time, virtually all pheromones known were those of insects. They predicted that the majority of pheromones should contain between 5 and 20 carbons and have molecular weights between 80 and 300. The major constraints in Bossert and Wilson's model were the volatility and diffusability of molecules. However, these predictions pertaining to the expected size of pheromone molecules were made at a time when the number of these compounds and the knowledge of their chemistry was quite limited. The subsequent discovery in phylogenetically diverse organisms of numerous semiochemicals that are waterborne, gustatory, or cutaneous in nature has laid to rest the idea held for so long that pheromones are highly volatile, low-molecular-weight compounds (Hadley, 1985).

General Conclusions. We have demonstrated that a series of synthetic saturated and monounsaturated methyl ketones elicit courtship behavior from male red-sided garter snakes. As in an earlier study (Mason et al., 1989), we find that the natural methyl ketones from the female are more attractive to males. In part this is due probably to the fact that only the major components of the methyl ketone series were tested.

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INFLUENCE OF LECTINS ON FEMALE SEX PHEROMONE RECEPTION BY *Heterodera schachtii* (NEMATODA: HETERODERIDAE) MALES

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Abstract—A bioassay was developed in which the chemotactic behavior of males of the sugarbeet cyst nematode *Heterodera schachtii* towards the female sex pheromone can be observed and documented at any time. The standardized test, performed under sterile conditions, gives reproducible results within 1–2 hr. Incubation of males with the lectins from *Canavalia ensiformis*, *Triticum vulgare*, *Arachis hypogaea*, *Helix pomatia*, and *Limax flavus* did not affect chemotactic attraction to the sex pheromone. Supplementing the bioassay medium with the lectin from *C. ensiformis* also had no effect. *C. ensiformis* lectin binding to the surface of the exudate produced by the secretory gland cell of the amphids, the main chemoreceptors, did not inhibit the passage of fluorochrome fluorescein isothiocyanate into the amphidial canals, where the receptor dendrites are localized. Amphidial exudate synthesis was enhanced during pheromone reception.

Key Words—Sugarbeet cyst nematode, *Heterodera schachtii*, Nematoda, bioassay, female sex pheromone reception, lectins.

INTRODUCTION

As in most insects and mites, the communication between the sexes of amphimictic species of root parasitic nematodes is based on the reception of female sex pheromones by males (e.g., Bone, 1987). The first compound with sex pheromone activity was recently isolated and identified from females of the soybean cyst nematode, *Heterodera glycines*, by Jaffe et al. (1989). Sex pheromone reception is obviously the main behavioral activity of cyst nematode males. Having emerged after the final molt from the saclike J_3 juvenile cuticle,

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they move through the soil in search of pheromone-producing females. Males do not feed, and they live from the energy reserves stored during their juvenile development. They orient themselves along concentration gradients of the sex pheromone (Green, 1980), and, having found a female, they try to copulate by coiling around the vulval area of the females.

Investigations on mating behavior, receptor physiology, and sex pheromone composition require a sensitive and reproducible bioassay under sterile conditions. The sugarbeet cyst nematode *Heterodera schachtii* is a good model for such studies as it can easily be reared on sterile root systems in agar culture (Wyss and Zunke, 1986).

With our bioassay, we examined the effect of lectins on female sex pheromone reception by males. Zuckerman (1983), Zuckerman and Jansson (1984), and Jansson (1987) proposed a hypothesis that sugar moieties in receptor-associated exudates of the amphids and inner labial sensilla (purportedly the main anterior chemosensilla of nematodes; see Wright, 1983) or on the receptor membranes (Jansson, 1987) may recognize chemoattractants. They suggested that chemoreception may be inhibited by lectins that bind to these carbohydrates and by enzymes that hydrolyze them.

Having examined carbohydrate-specific lectin binding to the amphidial exudates of *H. schachtii* males (Aumann and Wyss, 1987, 1989), we then tested the possible effect of these lectins (from *C. ensiformis*, *Triticum vulgare*, *Arachis hypogaea*, and *Helix pomatia*) as well as the *Limax flavus* lectin on female pheromone reception.

METHODS AND MATERIALS

Nematodes. Males of *Heterodera schachtii* were obtained from sterile root cultures of *Raphanus sativus* var. *oleiformis* cv. Pegletta grown at 25 ± 2°C in the dark in a nutrient agar medium (Dropkin and Boone, 1966). In this resistant oilradish cultivar nearly all nematodes develop into males. Care was taken to remove males only from dishes without females to exclude contact with the sex pheromone prior to the test. In addition, only very active males without any sign of food reserve depletion were selected for the bioassay.

Bioassay System. The pheromone solution used as a male attractant in the bioassay was obtained by transferring 100 white virgin females (mostly with adhering gelatinous matrix produced one to three days after the final molt) from sterile root cultures of *Sinapis alba* cv. Albatros or *Brassica rapa* var. *silvestris* f. *campestris* into a microcentrifuge tube filled with 1.7 ml of ultrapure water (Merck, Darmstadt, F.R.G.). The females were immediately stored at 4°C, and the supernatant was used 1–12 days later. The assay was performed in sterile 6-cm-diameter plastic Petri dishes coated with 1.5–2 ml of 1.5% autoclaved

agarose (Sigma, Deisenhofen, F.R.G., type I) in glass-distilled water. Ten microliters of the pheromone solution were pipetted on a 5-mm-diameter filter paper disk placed on the agarose surface in the center of the Petri dish. During the following 3 hr, the pheromone was allowed to diffuse into the agarose, forming a concentration gradient. A 7.5-mm-radius circle with its center in the center of the filter paper disk was marked on the bottom of the Petri dish. The distance between the circle and the filter margin was thus 5 mm.

For each test three males were transferred with a fine needle equidistantly onto the agarose above the circle. Their chemotactic behavior towards the pheromone was observed after 10, 30, 60, 90, and 120 min under a stereo microscope at 10× magnification. Filter paper contact of the males was defined as movement in the region covered by the disk or at least as head contact with it. Only active males that moved away from the point of inoculation were considered in the results. Those that stayed at this point have obviously been injured by the process of transfer. Nematode tracks on the agarose surface were visualized by lifting the Petri dishes at a determined angle to the epiillumination source of the stereo microscope or by exposing them on Kodak Kodalith Ortho type 3 graphic films (Ward, 1973). A possible effect of soluble filter paper compounds on nematode chemoreception was tested by a bioassay comparison of the sex pheromone with ultrapure water.

Lectin Treatments. Males were carefully removed from the sterile root cultures with a fine needle and transferred into lectin buffer where they were washed for at least 30 min. Phosphate-buffered saline (PBS), pH 6.8, with 0.1 mmol/liter $CaCl_2$ and $MnCl_2$ was used for the lectins from *Canavalia ensiformis* (Con A), *Triticum vulgare* (WGA), *Arachis hypogaea* (PNA), and *Helix pomatia* (HPA). The lectin from *Limax flavus* (LFA) was buffered in Tris-buffered saline (TBS), pH 8.0, with 10 mmol/liter $CaCl_2$. The fluorescein isothiocyanate (FITC) -conjugated lectins Con A, WGA, PNA, and HPA and the tetramethylrhodamine isothiocyanate (TRITC) -labeled Con A and WGA were purchased from Sigma. The FITC- or TRITC-labeled LFA was obtained from E.Y. Labs. (San Mateo, California). The carbohydrate specificities of these lectins are described by Goldstein and Poretz (1986). The males were incubated with the lectins (100 μg/ml) for 15 min at room temperatue in the dark. As preliminary bioassays indicated that only HPA may have an inhibitory effect on chemotaxis, a threefold concentration of this lectin was used also. After lectin incubation, the males were washed with PBS or TBS to remove unbound lectin. Controls were performed with heat denatured lectins (10 min in boiling water).

The duration of Con A binding to the amphidial exudates was examined by fluorescent microscopy (Aumann and Wyss, 1987) after the bioassay. The binding duration to males moving in the pheromone gradient was compared to males transferred to agarose without pheromone. In a second experiment, Con A (Sigma type VI) in a concentration of 1 mg/ml was mixed at 35–40°C into

the agarose. In this case, the bioassay was performed as described above, but without a lectin incubation of the males prior to the test. The behavior was observed under a stereo microscope as described under "Bioassay system." All results were analyzed with the chi-square test.

Lectin Effect on Fluorochrome Binding. A possible plugging effect of lectin binding to the amphidial exudate was tested by incubating PBS-washed nematodes with 200 μg/ml TRITC-conjugated Con A for 30 min at room temperature in the dark. Then the nematodes were washed three times with PBS and incubated for 30 min under the same conditions with 400 μg/ml FITC (Merck). After three washes with PBS, the nematodes were examined by fluorescent microscopy with different filter systems for TRITC and FITC (Aumann and Wyss, 1987). Nematodes incubated with Con A only served as controls.

RESULTS

The effect of male incubation with different lectins on sex pheromone reception is shown in Table 1. The lectin treatments (Con A, WGA, PNA, HPA, and LFA) did not differ significantly from the heat-denatured lectins used

TABLE 1. LECTIN INCUBATION EFFECTS ON FEMALE SEX PHEROMONE RECEPTION BY *H. schachtii* MALES

Treatment	No. of males tested	No. of males with filter paper contact				
		10 min	30 min	60 min	90 min	120 min[a]
Con A 100 μg/ml	83	4a[b]	38 a	64 a	72 a	75 a (90.4)
Con A denatured	82	6 a	47 a	69 a	68 a	74 a (90.2)
WGA 100 μg/ml	87	11 a	52 a	73 a	72 a	73 a (83.9)
WGA denatured	86	5 a	51 a	64 a	69 a	69 a (80.2)
PNA 100 μg/ml	80	11 a	43 a	57 a	60 a	66 a (82.5)
PNA denatured	75	8 a	41 a	60 a	62 a	64 a (85.3)
HPA 100 μg/ml	58	8 a	36 a	51 a	53 a	52 a (89.7)
HPA denatured	55	3 a	31 a	47 a	46 a	46 a (83.6)
HPA 300 μg/ml	55	5 a	31 a	39 a	45 a	49 a (89.1)
HPA denatured	57	7 a	36 a	46 a	52 a	55 a (96.5)
LFA 100 μg/ml	56	6 a	37 a	46 a	53 a	53 a (94.6)
LFA denatured	58	4 a	37 a	51 a	56 a	54 a (93.1)

[a] Attraction rates (in percent) at the end of the bioassay (120 min) are given in parentheses.
[b] Values with a same letter for each treatment and observation time are not significantly different ($P \leq 0.05$) according to the chi-square test.

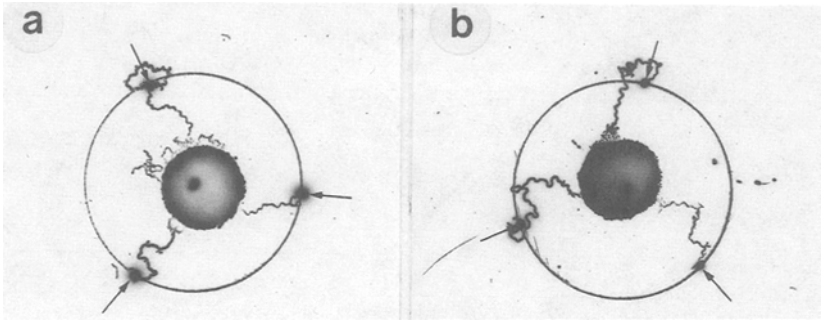


FIG. 1. Movement of *Heterodera schachtii* males in the female sex pheromone gradient: (a) PNA-treated males, (b) males treated with heat-denatured PNA. The initial positions of males are indicated by arrows.

as controls. Even the threefold concentration of HPA (300 μg/ml) did not significantly affect pheromone reception at any observation time. The attraction rates at the end of the bioassay ranged between a minimum of 80.2% (denatured WGA) and a maximum of 96.5% (denatured HPA). Differences between lectin treatments and controls were not significant. Typical bioassay plates with visible nematode tracks are shown in Figure 1. A comparison of sex pheromone with ultrapure water showed that the water-treated filter disks had no effect on male attraction. Of a total of 47 and 46 males examined, 80.8% and 2.2% had contacted the filter disks treated with pheromone and ultrapure water, respectively, after 120 min.

The effect of performing the bioassay in agarose supplemented with Con A is shown in Table 2. Significant differences between Con A–agarose and agarose without the lectin were not observed at any time.

Table 3 presents observations on the duration of lectin binding to the amphidial exudate when males were treated with Con A. The comparison between males that had moved in the pheromone gradient and in agarose only

TABLE 2. EFFECT OF CON A–AGAROSE ON FEMALE SEX PHEROMONE RECEPTION BY *H. schachtii* MALES

Bioassay medium	No. of males tested	No. of males with filter paper contact				
		10 min	30 min	60 min	90 min	120 min
Con A–agarose	65	5 a[a]	38 a	53 a	56 a	53 a
Agarose only	64	6 a	30 a	44 a	49 a	51 a

[a] Values with a same letter for each bioassay medium and observation time are not significantly different ($P \leq 0.05$) according to the chi-square test.

TABLE 3. CON A BINDING TO AMPHIDIAL EXUDATE OF *H. schachtii* MALES DEPENDING ON TIME OF MOVEMENT IN PHEROMONE GRADIENT OR AGAROSE ONLY

Treatment	No. of males tested	Fluorescence on surface of amphidial exudate	
		Visible	Not visible
3-hr pheromone a[a]	37	5	32
3-hr agarose only b	31	28	3
6-hr agarose only c	19	12	7

[a]Treatments with a same letter are not significantly different ($P \leq 0.05$) according to the chi-square test.

shows that most males had lost the amphidial fluorescence after 3 hr in the pheromone but not in agarose. The differences between 3-hr pheromone, 3-hr agarose and 6-hr agarose were significant.

FITC binding sites after Con A incubation are shown in Table 4. In every specimen examined, Con A bound only to the surface of the amphidial exudate, whereas FITC labeled the anterior nerve system and the entire exudate, also within the amphidial canals.

TABLE 4. FITC BINDING SITES AFTER TRITC–CON A INCUBATION OF *H. schachtii* MALES

Treatment	No. of males tested	Con A binding to surface of amphidial exudate	FITC binding sites	
			Amphidial exudate including amphidial canal	Anterior nerve system
TRITC-Con A followed by FITC	48	48	48	48
TRITC-Con A only	49	49		

DISCUSSION

The bioassay for female sex pheromone described here has several advantages compared to other bioassays using cyst nematodes (Green, 1966, 1967; Greet et al., 1968; Green and Plumb, 1970; Evans, 1970; Green and Greet; 1972, Rende et al., 1982; Huettel and Rebois, 1986; Bone, 1986). It requires at most 2 hr to demonstrate pheromone-directed movement of males to the

attractant source. Attraction rates were high and the pheromone source was homogeneous for each experiment. The bioassay is sensitive and reproducible and may be performed under similar sterile conditions with other cyst nematode species.

Lectin effects on chemoreception by nematodes were first described by Jansson and Nordbring-Hertz (1984). They demonstrated that treatment of the bacteriovorous nematode *Panagrellus redivivus* with 25 μg/ml of the lectin from *Limulus polyphemus* (LPA) reduced chemotaxis to the nematophagous fungus *Drechmeria coniospora*. Bone and Bottjer (1985) observed an inhibition of female sex pheromone reception after males of the gastrointestinal animal parasite *Trichostrongylus colubriformis* had been treated with 20 μg/ml of the lectin from *Lens culinaris* (LCA). Jeyaprakash et al. (1985) also reported a partial attraction inhibition of the bacteriovorous nematode *Caenorhabditis elegans* to *Escherichia coli* filtrates after Con A and LPA (200 μg/ml) treatment. As a possible explanation for chemotaxis inhibition by lectins, Bone and Bottjer (1985) suggested that LCA may bind either to the nematode receptors or in their vicinity, causing a physical hindrance in their function. Jeyaprakash et al. (1985) proposed that lectins binding to sugars on sensillum exudates may partially plug the pores of the sensillum. The hypothesis proposed by Zuckerman and Jansson (1984) and Jansson (1987) suggests that sugar compounds of the exudate are the same as the terminal sugars of the chemoreceptors located on the sensilla membranes and that lectins binding to the exudate may thus also block the receptor molecules. This implies that the exudate is permeable to lectins.

Two electron microscopic studies on lectin binding to amphidial exudates have shown that in root parasitic nematodes lectins bind only to the surface of the exudate (Forrest and Robertson, 1986, McClure and Stynes, 1988). Similarly, we were never able to detect fluorochrome-conjugated lectins in the amphidial canals of *H. schachtii* males. Only in dead males did lectin binding become evident at these sites (Aumann and Wyss, 1987). It would indeed be most surprising if in living, exudate-producing nematodes, the large lectin molecules with a molecular mass of at lest 36 kDa could reach the receptor membranes.

The suggestion that lectin binding to the surface of the exudate may partially plug the pores of the chemoreceptor sensilla (Jeyaprakash et al., 1985) and hence inhibit chemoreception appears questionable for root parasitic nematodes. We showed that binding of Con A (Mr 102 kDa) to the surface of the amphidial exudate of *H. schachtii* males does not inhibit the passage of FITC (Mr 389.4) through the exudate and its subsequent binding to the anterior nerve system (Table 4). Chemoattractants of similar low molecular mass, such as vanillic acid (Mr 168.1), the compound with sex pheromone activity in *Heterodera glycines* females (Jaffe et al., 1989), thus may easily pass through gaps between the much larger lectin molecules that bind to the exudate surface.

Treating males with Con A, WGA, PNA, and HPA, all with a very strong affinity for surface sugar moieties on the exudate surfaces of the anterior amphidial and posterior spicular sensilla (Aumann and Wyss, 1987, 1989) clearly had no inhibitory effect on pheromone reception under sterile in vitro conditions (Table 1). Even supplementing the agarose medium with Con A was not effective (Table 2). Similarly the chemotactic stimulus of possibly low-molecular-mass attractants from potato root diffusates could not be inhibited by treating J_2-juveniles of the potato cyst nematode *Globodera rostochiensis* with 250 μg/ml Con A (Forrest et al., 1988). Con A treatment (200 μg/ml) of J_2-juveniles of *Meloidogyne* spp. also had no effect on soybean root penetration (Davis et al., 1989). Kaplan and Davis (1989) even reported that treatment of *Radopholus citrophilus* with 100 μg/ml of Con A, WGA, soybean agglutinin, and the lectin from *Lotus tetragonolobus* stimulated an 80% increase of infection rates of citrus root segments.

The hypothesis by Zuckerman (1983) and Jansson (1987), which implies that lectins with a binding capacity to sugars on the receptor-associated exudates or on the receptor membranes may inhibit chemoreception, in part confirmed by in vitro experiments (see above), can obviously not be applied to all nematodes. We suppose that the exudates secreted by chemosensory sensilla of root parasitic nematodes may have a protective function. They may act like a filter, allowing the passage of low-molecular-mass chemoattractants to the receptors on the membranes of the dendritic processes within the receptor cavity of the chemosensilla, but preventing macromolecules, such as enzymes, from reaching the receptors. The amphidial exudate of *H. schachtii* males is resistant to many enzymes (Aumann, 1989).

Our observation that lectin binding to the amphidial exudate disappears more rapidly on males moving in the pheromone gradient than on agarose only (Table 3) also indicates that exudate production is stimulated by the interaction of chemoreceptors with female sex pheromone molecules. The enhanced exudate production may be connected with mechanisms for the removal of receptor proteins with a high turnover rate and/or inactivation of pheromone molecules after their reception on the sensilla membranes.

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ECOLOGICAL IMPLICATIONS OF CONDENSED TANNIN STRUCTURE: A CASE STUDY

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Abstract—Condensed tannins were isolated from bitterbrush (*Purshia tridentata*) and blackbrush (*Coleogyne ramosissima*). Structural analyses showed that both tannins were procyanidins of similar polymer length. The overall stereochemistries at C-3 and C-4, however, differed between the two tannins. These changes in stereochemistry resulted in blackbrush tannins being less preferred than bitterbrush tannins when offered to snowshoe hares (*Lepus americanus*). It is unlikely that differences in protein-precipitating abilities are the cause for the preference of the bitterbrush over the blackbrush tannins. Instead, we hypothesize that condensed tannins may be depolymerized and absorbed following ingestion. Differences in tannin structure can lead to different depolymerized products and rates of depolymerization, both of which may affect herbivore preferences.

Key Words—condensed tannin, procyanidin, blackbrush, bitterbrush, plant defense.

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INTRODUCTION

Condensed tannins have traditionally been thought to reduce plant preference by digestion inhibition (Feeny, 1969, 1976; Rhoades and Cates, 1976; Swain, 1979) because they can complex and render inactive digestive enzymes (Swain, 1979) and precipitate dietary proteins (Feeny, 1969), making them less easily degraded. Work on condensed tannin–protein interactions has stressed protein structure (Asquith and Butler, 1986; Hagerman and Butler, 1981; Hagerman and Klucher, 1986; Mehansho et al., 1987), pH (Hagerman and Butler, 1981; Hagerman and Klucher, 1986; Van Sumere et al., 1975), tannin–protein concentrations (Hagerman and Robbins, 1987; McManus et al., 1981), tannin molecular weight (Porter and Woodruffe, 1984; Jones et al., 1976; Takechi et al., 1985) and tannin source (Asquith and Butler, 1986). Although Zucker (1983) proposed that tannin structure may play a major role in tannin–substrate interactions, no investigations have dealt with condensed tannin structure and its effect on plant–animal interactions.

To test Zucker's (1983) proposal that condensed tannin structure has biological implications for herbivory, we chose tannins from two related plants from the family Roseaceae, blackbrush and bitterbrush. Blackbrush is a low-quality food for herbivores (Provenza and Malechek, 1984), and its current season growth (CSG) is avoided by goats because of its condensed tannin content (Provenza et al., 1989). Bitterbrush, on the other hand, is an important food source for many mammalian herbivores (Sheperd, 1971; Kufeld, 1973; Kufeld et al., 1973), even though its condensed tannin level appears to be similar to that of blackbrush (see Results). Hence, tannins from these two plants seemed to be logical choices for testing the hypothesis that tannin structure affects its ecologically relevant biological properties.

METHODS AND MATERIALS

[^{13}C]NMR Spectra. [^{13}C]NMR spectra of condensed tannins (500 mg in 2.0 ml d_6-acetone–water, 1 : 1 v/v) were taken on a JEOL FX90Q instrument. Chemical shifts were measured from the d_6-acetone methyl peak (29.8 ppm relative to TMS) and are reported relative to TMS.

Optical Rotations. Optical rotations were measured at the sodium D line on a Perkin Elmer model 241 spectropolarimeter at 25°C in aqueous methanol (50%). Tannin concentrations were about 0.5 mg/ml.

Isolation and Characterization of Condensed Tannins. Condensed tannins from CSG of winter-dormant blackbrush and bitterbrush twigs were isolated using a modification of Hagerman and Butler's (1980) procedure in which the back extraction of the tannins with liquefied phenol was omitted. Tannins from

400-g batches of twigs were pooled until sufficient tannin was isolated for bioassays and structural investigations.

Tannin purity was confirmed by both HPLC (Mueller-Harvey et al., 1987) and [^{13}C]NMR analyses (Porter et al., 1982; Czochanska et al., 1980). Qualitative analysis for procyanidins and prodelphinidins was conducted by paper chromatography of BuOH–HCl–Fe^{2+}-treated tannins using reported R_fs and colors (Mole and Waterman, 1987). Stereochemical assignments were based on [^{13}C]NMR spectra (Porter et al., 1982; Czochanska et al., 1980), optical rotations (Czochanska et al., 1980) and by HPLC analyses of the acid-catalyzed depolymerized products (determined by their retention times) (Shen et al. 1986) formed in the presence of α-toluenethiol (Figure 1).

Protein precipitation capacity of each tannin was determined by Hagerman's (1987) radial diffusion method using bovine serum albumin (BSA) as protein. Ten microliters of a standard solution of purified tannins, 50 mg tannin/0.500 ml aqueous acetone (1 : 1 v/v), was used for both bitterbrush and blackbrush tannins. Sixteen astringency measurements were made for each tannin.

Bioassays with Hares. Captive snowshoe hares were offered a choice

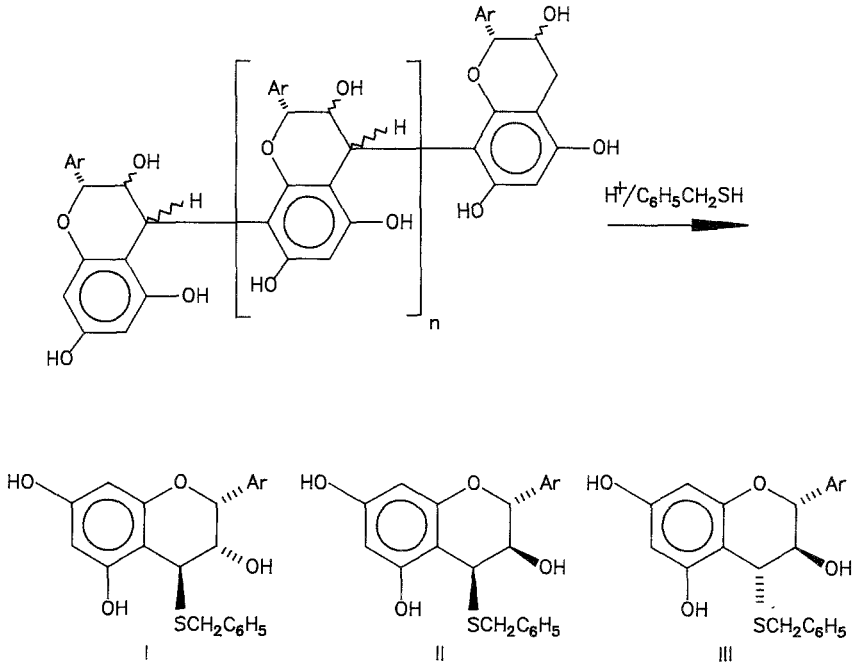


FIG. 1. Products from the oxidatively depolymerized procyanidin extender units. Terminal unit produces catechin or epicatechin.

between oatmeal containing 3% (dry wt) of either purified blackbrush or bitterbrush tannin, approximately the concentration of each tannin isolated from each plant (see Results). Hares had been exposed to these diets previously. Thus hares may have learned to avoid, based on previous consequences, or prefer one diet over another (Provenza et al., 1989). Tannins were applied by mixing with slightly dampened oatmeal. After freeze-drying, 40 g of each diet was offered to eight individually caged hares. No untreated oatmeal was offered. After 10 hr, uneaten oatmeal was collected and weighed.

RESULTS

Both bitterbrush and blackbrush provided condensed tannins as light tan, fluffy powders in about 3.0 and 2.5% yield (dry weight), respectively, when using Hagerman and Butler's (1980) procedure. Hence, both plants contained similar amounts of extractable condensed tannin polymer. The levels of nonextractable tannin and low-molecular-weight oligomers were not determined.

HPLC (Mueller-Harvey et al., 1987) and [^{13}C]NMR (Czochanska et al., 1980; Porter et al., 1982) analyses indicated that the isolated tannins were pure condensed tannins. Paper chromatography (Mole and Waterman, 1987) showed only cyanidin as a product when either tannin was treated with BuOH–HCl–Fe^{2+}.

The [^{13}C]NMR spectra (Figure 2) showed differences between the two tannins. In particular, the stereochemistries at C-3 (catechin vs. epicatechin) and C-4 vary quantitatively between the two tannins (Table 1). These values were calculated by integration of the corresponding signals, a valid method (Porter et al., 1982), but nonetheless imprecise due to incompletely resolved peaks and baseline noise.

The above [^{13}C]NMR interpretations concerning catechin–epicatechin ratios were confirmed qualitatively by HPLC analyses of the depolymerized tannins (Table 2). This method could not be used to quantitatively analyze the stereochemistry at C-4 because this is the site of reaction.

Average molecular weights of the two isolated tannins were calculated (Czochanska et al., 1980) from their [^{13}C]NMR spectra by integration of the signals at 73 ppm (C-3 extender units) and 67 ppm (C-3 terminal unit). The molecular weights of the two isolated tannin fractions are similar (Table 1), although precise integrations again are not possible due to baseline noise and unresolved peaks.

The specific rotations, $[\alpha]_{589}$, of bitterbrush and blackbrush tannins were $-49.95° \pm 0.05$ and $+42.33° \pm 0.55$, respectively.

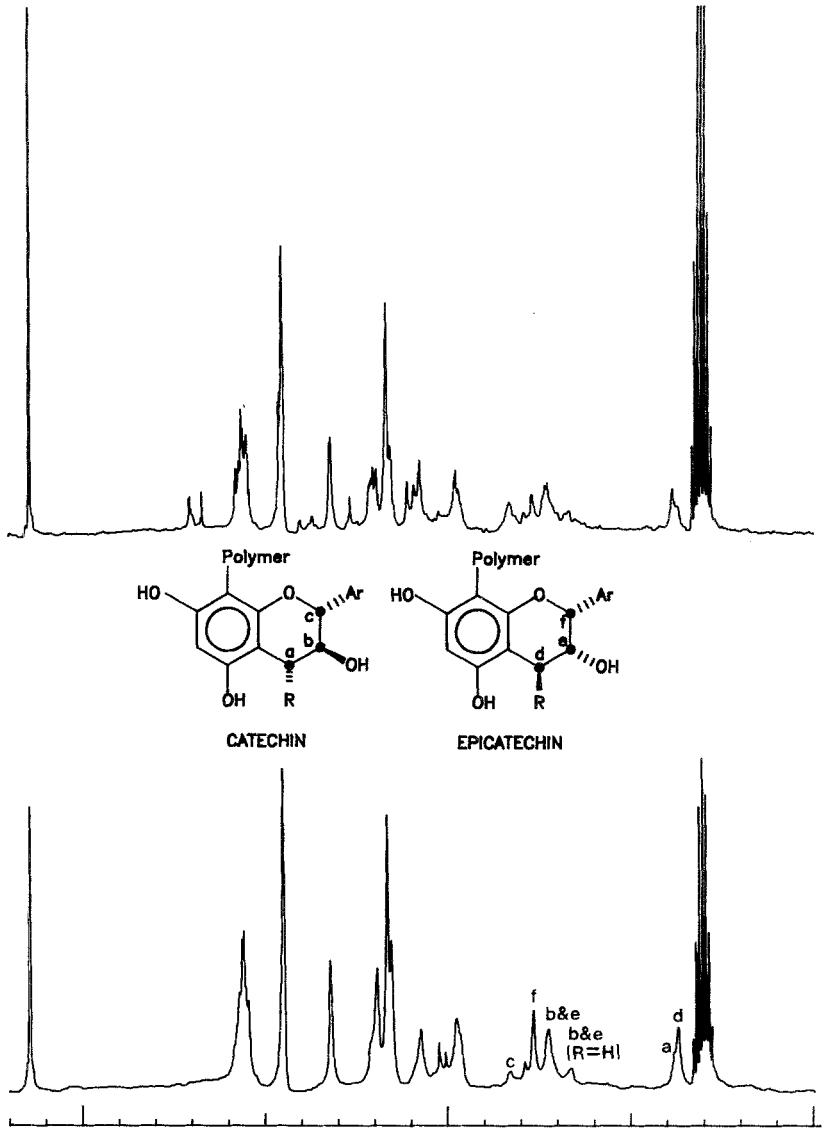


FIG. 2. [^{13}C]NMR spectra of purified bitterbrush (upper) and blackbrush (lower) tannins. Pertinent peak assignments are shown on the lower spectrum. The scale is from 0 to 220 ppm (10 ppm increments).

TABLE 1. CALCULATED (BASED ON [^{13}C] NMR SPECTRA) DISTRIBUTIONS OF STEREOCHEMISTRIES AT C-3 AND C-4,[a] AND AVERAGE MOLECULAR WEIGHTS OF BITTERBRUSH TANNINS

	Catechin/ epicatechin[b]	C-4(*cis*)/ C-4(*trans*)[c]	Avg. molecular weight[d]
Bitterbrush	55:45	55:45	1300
Blackbrush	20:80	20:80	1400

[a] *cis/trans* is relative to the B ring.
[b] Calculated by the ratio of area(83ppm)/area(77ppm).
[c] Calculated by the ratio of area(37ppm)/area(38ppm).
[d] Calculated by the formula: 288[area(73ppm)/area(67ppm) + 1].

TABLE 2. PRODUCT DISTRIBUTIONS OF DEPOLYMERIZED PROCYANIDINS FROM BLACKBRUSH AND BITTERBRUSH[a]

	I	II	III	Catechin/ epicatechin[b]
Bitterbrush	3.92	1	3.53	54:46
Blackbrush	4.88	1.40	1	33:67

[a] Compounds I, II, and III correspond to those found in Figure 1.
[b] Calculated by the formula (II + III)/I.

Snowshoe hares showed about a threefold preference ($P = 0.024$; Wilcoxon rank sum test) for the bitterbrush tannin over the blackbrush tannin (Table 3). Only seven of the eight hares used in the bioassay are reported in Table 3 because one hare did not consume either diet.

Bitterbrush tannins appeared more astringent ($P < 0.01$, paired t test; but see Discussion) in Hagerman's (1987) radial diffusion assay than blackbrush tannins. Based upon the average of 16 assays for both tannins, the difference was not large; the area of bitterbrush-tannin precipitated proteins was 1.28 $\pm$ 0.15 (standard deviation) times greater than the area of blackbrush-tannin precipitated proteins.

DISCUSSION

Blackbrush tannins are comprised predominantly of epicatechin units (R configuration at C-3) whereas bitterbrush tannins are comprised of about a 55:45 mixture of catechin–epicatechin units (Tables 1 and 2). While the catechin–

TABLE 3. RESULTS OF FREE CHOICE FEEDING BIOASSAY OF BLACKBRUSH- VERSUS BITTERBRUSH-TREATED OATMEAL

Hare	Amount of treated oatmeal consumed		Preference index[a]
	Bitterbrush tannin	Blackbrush tannin	
1	30.81	3.80	0.89
2	8.28	1.85	0.82
3	33.89	10.57	0.76
4	21.41	0.18	0.99
5	33.91	33.55	0.50
6	18.62	2.75	0.87
7	3.69	8.03	0.31
Average			0.73
Standard error			0.09

[a] Preference index = bitterbrush diet consumed/total diet consumed. A value of 0.50 implies no discrimination between diets; values greater or less than 0.50 imply a preference or avoidance of bitterbrush over blackbrush tannins respectively.

epicatechin estimate varies from 20:80 (^{13}C]NMR) to 33:67 (thiolysis) in blackbrush, the latter value is suspect because thiolysis reactions of proanthocyanidins are not quantitative (Karchesy and Hemingway, 1980). In addition, based on results of Czochanska et al. (1980), the specific rotations that we observed correspond to a catechin–epicatechin ratio of 55:45 and 20:80 for bitterbrush and blackbrush tannins, respectively, in agreement with the [^{13}C]NMR estimate. Hence based on both the [^{13}C]NMR and optical rotation analyses, bitterbrush has a catechin–epicatechin ratio that is five times that of blackbrush. The stereochemistry at C-4 also differs between the two tannins (Table 1). This latter result is not surprising because the stereochemistry at C-3 (catechin vs. epicatechin) is a strong predictor of the stereochemistry at C-4 with naturally occurring procyanidins (Fletcher et al., 1977).

The blackbrush tannins inhibit food ingestion by snowshoe hares more than bitterbrush tannins. Both tannin types were highly purified polymers; no contaminants were evident from either the [^{13}C]NMR spectra or HPLC chromatographs. Difference in tannin structure is the only explanation for the difference in intake of the two diets.

The differences in predominant stereochemistry at C-3 and C-4 of bitterbrush and blackbrush tannins lead to differences in preferred conformations (Fletcher et al., 1977). Zucker (1983) predicted that changes in both stereochemistry and conformation will lead to selective abilities to complex specific proteins. Our study is the first to confirm this prediction. Specifically, the struc-

tural differences in the two tannins investigated lead directly to differences in the physical and/or chemical properties of the resultant tannin–protein complexes. Others (Asquith and Butler, 1985, 1986; Blytt et al., 1988) also have reported that condensed tannins from different plant sources have varying abilities to complex proteins or inhibit enzymes but have not described the structural differences of the tannins.

The differences in tannin structures in this study are based on stereochemical assignments at C-3 and C-4, although other minor structural differences, such as linkage isomerism (C-4–C-8 versus C-4–C-6) (Hemingway et al., 1982; Nonaka et al., 1981) may exist. Condensed tannins often display much larger diversities in structures, including oxygenation patterns on the A ring (procyanidins, 5-deoxyprocyanidins) and B ring (procyanidins, prodelphinidins, propelargonidins), linkages (C-4–C-8, C-4–C-6, A type tannins), molecular weight profiles (Williams et al., 1983), branching (hypothesized by Porter, 1986), enantiomerism (Ellis et al., 1983), and substituents (Hsu et al., 1985). Because our study showed that minor differences in tannin structures can lead to changes in both tannin preferences and tannin–protein interactions, comparison of other tannins whose structural differences are more pronounced is expected to show even greater diversity in their biological functions.

The preference for bitterbrush tannin by snowshoe hares, coupled with bitterbrush tannin's ability to precipitate a larger area of BSA protein, appears contradictory to the hypothesis that tannin–protein complexes are the cause of herbivore avoidance of tannins. We propose three possible explanations for this apparent contradiction. First, BSA protein may not be representative of the proteins "targeted" by tannins, and certain enzymatic proteins critical to digestion may exhibit qualitatively different activities if used in the radial diffusion assay. Second, the area measured in the radial diffusion assay is also a function of the diffusion characteristics of tannin–protein complexes. Hence, our results could be consistent with bitterbrush tannins having less binding ability with proteins than blackbrush tannins if the bitterbrush tannin–protein complexes diffuse to a greater extent.

While these two explanations for our results are feasible and deserve further investigation, we propose as a likely alternative explanation that condensed tannins deter herbivore feeding by acting as toxins and not as digestion inhibitors by protein precipitation. Other researchers have obtained data that suggest the toxic nature of tannins (Robbins et al., 1987; Butler et al., 1986; Provenza et al., 1989; Lindroth and Batzli, 1984) and question the validity of tannins acting as digestion inhibitors (Blytt et al., 1988; Butler et al., 1986, and references therein).

For condensed tannins to be toxic, they must be depolymerized before they can be absorbed into the body. While not generally appreciated, many condensed tannins are easily depolymerized under a variety of mild conditions, not

unlike those found in many digestive tracts. For instance, Porter et al. (1985) report that the lability of the interflavane bonds of certain tannins under acidic conditions is similar to that of the glycosidic bond of phenol glycosides.

Both the rate of depolymerization and the products formed are dependent on tannin structure. For example, procyanidins can be depolymerized under mild acidic conditions (0.6 N HCl; 30°C; Fletcher et al., 1977) whereas the 5-deoxyprocyanidins (Hemingway, personal communication), and proanthocyanidin-A2 tannins (Jacques and Haslam, 1974) are highly resistant to depolymerization. For procyanidins, 2,3-*cis*-procyanidins (C-4–C-8 linkages) undergo stereospecific depolymerization more rapidly than their 2,3-*trans* isomers (Hemingway and McGraw, 1983; Hemingway et al., 1983). Roux et al. (1980) suggest that the kinetic control exhibited in these latter reactions is the result of steric (and possibly neighboring-group participation) considerations and that the use of vigorous conditions may explain why some researchers (for instance, Fletcher et al., 1977) have failed to observe this kinetic control. These considerations suggests that blackbrush tannins, which are mainly comprised of 2,3-*cis* units, should depolymerize more rapidly than bitterbrush tannins when ingested by herbivores. Hence, the preference by snowshoe hares of bitterbrush tannins over blackbrush tannins may simply be a result of bitterbrush tannins undergoing slower depolymerization/absorption in the digestive tract, leading to a lower degree of toxicity.

CONCLUSIONS

We have demonstrated for the first time that condensed tannin structure is an important determinant of biological activity. Specifically, both tannin–protein interactions and relative tannin preference by snowshoe hares are dependent on the stereochemistry at C-3 and/or C-4 of procyanidins. It is expected that investigations of more diverse tannin structures will demonstrate even more dependence of biological effects on tannin structure.

In addition, we propose that the avoidance of blackbrush over bitterbrush tannins may be the result of blackbrush tannins depolymerizing in the digestive tract at a higher rate than bitterbrush tannins. Hence, ingestion of blackbrush tannin would lead to a faster and perhaps more complete absorption of toxic phenols than ingestion of bitterbrush tannins.

The multitude of both herbivores and tannin structures makes it impossible to generally assess the consequence of any given herbivore eating a plant rich in any given condensed tannin. Before such generalizations can be realized, researchers studying plant–herbivore interactions must critically examine the dogmas concerning the consequences of tannin ingestion in the gut (digestive inhibition, generalized defenses, not metabolizable) by conducting studies using purified condensed tannins with at least partially defined structures.

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DEVELOPMENT OF PHEROMONE LURE FOR MONITORING FIELD POPULATIONS OF *Eoreuma loftini* (Lepidoptera: Pyralidae)[1]

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Abstract—Formulations of Mexican rice borer (MRB) pheromone components were more effective in capturing males in fields of sugarcane when dispensed from rubber septa than when evaporated from four other substrates. Concentrations of 0.63-10.0 mg of total pheromone per lure were effective over a 112-day period when formulated in rubber septa in a ratio of 8:1:1.3 of (Z)-13-octadecenyl acetate, (Z)-11-hexadecenyl acetate, and (Z)-13-octadecenal, respectively. Component ratios of 4:1:1.3 and 8:1:1.3 were equally effective in capturing male MRB, but efficacy decreased with higher relative concentrations of (Z)-13-octadecenyl acetate.

Key Words—pheromone, lure, attractant, *Eoreuma loftini*, Mexican rice borer, Lepidoptera, Pyralidae, monitoring, development, populations.

INTRODUCTION

The Mexican rice borer (MRB), *Eoreuma loftini* (Dyer), was found in sugarcane fields in the lower Rio Grande Valley of Texas in the summer of 1980 (Fors, 1981; Fors and Aberca, 1983; Johnson and Van Leerdam, 1981). MRB caused yield losses in the 1980 and 1981 growing season (Fors, 1981) and also were found on several alternate hosts (Johnson and Van Leerdam, 1981). The presence of a female sex pheromone in MRB was demonstrated by Brown et

[1] Mention of a commercial or proprietary product does not constitute an endorsement by the USDA.

al. (1988), and they reported on the response of male MRB to ovipositor extracts prepared from virgin females. Three chemicals isolated from ovipositor rinses of virgin female MRB were identified as (Z)-13-octadecenyl acetate (Z-13-ODAc), (Z)-11-hexadecenyl acetate (Z-11-HDAc) and (Z)-13-octadecenal (Z-13-ODAl) in the approximate ratio of 8:1:1.3, respectively (Shaver et al., 1988). Traps baited with these chemicals in the natural ratio dispensed from filter paper captured as many male MRB as traps baited with three virgin females.

The initial reports (Shaver et al., 1988) demonstrated the efficacy of the pheromone components dispensed from filter paper. However, in practical application for use in traps for monitoring the presence and density of naturally occurring field populations of MRB, the pheromone should be formulated as bait in a slow-release substrate so it will be effective over extended periods and not require replacement on a daily basis. The objective of this study was to develop an effective lure in a dispensing substrate with extended longevity for MRB and to determine optimum component ratios and concentrations for use as bait in traps.

METHODS AND MATERIALS

Z-13-ODAc was either prepared in the laboratory (T.N.S.) by reduction of (Z)-13-octadecenal and subsequent acetylation of the alcohol (Shaver et al., 1988) or was purchased from Provesta Corp. (Bartlesville, Oklahoma 74004). Both preparations were isomerically pure (>99.9% *cis* isomer) and contained ca. 2% of other volatiles as measured by capillary gas chromatography using 50-m × 0.32-mm-ID vitreous silica OV-1701 column (SGE, Austin, Texas) or 50-m × 0.25-mm-ID vitreous silica Silar 10C column (Alltech Associates, Deerfield, Illinois). Z-13-ODAl was obtained from Aldrich Chemical Company (Milwaukee, Wisconsin) and was greater than 97% pure as measured by capillary GLC on the above columns. Z-11-HDAc was obtained from Sigma Chemical Co. (St. Louis, Missouri) and was greater than 98% pure as measured by capillary GLC.

Comparison of Dispenser Types. Five slow-release formulation dispensers were prepared to contain 5 mg each of an 8:1:1.3 mixture of Z-13-ODAc, Z-11-HDAc and Z-13-HDAl, respectively. Rubber septa (5 × 9 mm, sleeve type, 1171 rubber, West Co., Lancaster, Pennsylvania) were impregnated with pheromone by adding 250 μl of a 20 mg/ml solution of the three-component mixture in hexane in the large well of the rubber septa and allowing hexane to evaporate, after which an additional 250 μl of hexane was added and evaporated. Black polyvinyl chloride (PVC) plastic rod dispensers (Hendricks, 1983) were prepared by mixing neat samples of the three pheromone components at

a ratio of 8:1:1.3 in liquid vinyl chloride homopolymer to give 8 mg pheromone blend per gram of vinyl chloride. The pheromone–vinyl chloride blend then was poured into 6-mm glass tubing and heated to 145°C for ca. 15 min to polymerize the vinyl chloride. The impregnated PVC rods were extruded from the glass tubing after cooling in an ice-water bath and cut into 2.5-cm lengths that weighed ca. 625 mg and contained 5 mg of pheromone each. Two formulations of multilayered polymeric dispensers (laminated Hercon plastic pheromone dispensers) were prepared by Hercon Laboratories (New York). Vial dispensers were prepared by dispensing 62.5 μl of a hexane solution containing 80 mg/ml of an 8:1:1.3 blend of the three pheromone components into a 2-ml white, hard plastic vial with attached snap lid (Mitchell, 1979). The check bait consisted of three caged virgin females.

Pherocon IC (Trece Inc., Salinas, California 93915) traps were used in all field studies to evaluate the various slow-release dispensers. The traps were placed 50 m apart and 5 m inside the sugarcane fields at 1.5 m above ground level. Tests were run three times with three replicates of each dispenser type per run. The five slow-release formulation dispensers and check bait were randomized on the first night. Traps were then serviced and rotated daily for a period of 18 days, or three complete rotations, within each replicate. All lures were suspended within the Pherocon 1C traps about 2 cm above the bottom surface. Virgin females were placed in a 2.5 × 2.5 × 2.5-cm screen cage that also was suspended 2 cm above the bottom surface of the trap.

Comparison of Pheromone Concentration. Rubber septa were selected for determining the effect of pheromone concentration in a slow-release formulation on efficacy of the lure in field evaluations since they were the most effective substrate used in the first test (described above). Rubber septa were impregnated with the MRB pheromone to contain concentrations of the pheromone ranging from 0.15 to 10 mg/rubber septum. Universal moth traps (Shaver et al., 1990) (Great Lakes 1PM, Vestaburg, Michigan) were used in all comparisons of pheromone concentration. In an initial experiment, septa were impregnated with an 8:1:1.3 mixture of MRB pheromone to give six concentrations of pheromone ranging from 0.6 to 10 mg per septum. Traps were placed 50 m apart and 5 m inside sugarcane fields and about 0.5 m above ground level to the opening of the trap. Traps were rotated daily within each replicate. During each daily servicing, captured moths were removed from the traps and counted. This test was replicated three times and was run for 96 consecutive nights during July–October 1987, with 16 complete rotations of lures within each replicate. In the second test, septa were prepared to contain eight concentrations of the pheromone ranging from 0.15 to 10 mg of MRB pheromone and were placed in sugarcane fields as described in the first test. The test was run for 112 days during August–December 1987, with 14 complete rotations of each replicate. At the end of the 112-day test period, 1.25-mg and 5.0-mg lures from the same batch, but which

had been tightly sealed and stored in a freezer (−35°C), were used as bait in traps for a 10-day period. These were compared for MRB capture with lures containing the same original pheromone concentrations but that had been aged under field conditions for 112 days.

Comparison of Pheromone Component Ratios. Pheromone component ratios were compared by impregnating rubber septa with 5 mg total pheromone components blended in five ratios ranging from 4:1:1.3 to 60:1:1.3 of Z-13-ODAc, Z-11-HDAc and Z-13-ODAl, respectively. The relative concentration of Z-11-HDAc and Z-13-ODAc remained constant while varying relative concentrations of Z-13-ODAc. Universal bucket traps were suspended from 6.5-mm-diameter reinforcement rods ca. 50 cm above ground level around the perimeter of sugarcane fields at ca. 30-m intervals. Each replicate was randomized initially, and subsequently traps were rotated within individual replicates each time traps were checked for moth capture at two- to three-day intervals.

RESULTS AND DISCUSSION

Comparison of Pheromone Dispensers. Pheromone 1C traps baited with MRB pheromone, 8:1:1.3 ratio of Z-13-ODAc and Z-11-HDAc and Z-13-ODAl, respectively, impregnated into rubber septa at 5.0 mg total pheromone per septum captured as many male MRB as similar traps baited with three virgin females (Table 1). There was no apparent loss of activity in the rubber septa during the three-week test period. Traps baited with the same concentration and ratio of pheromone chemicals formulated in molded black PVC rods captured

TABLE 1. COMPARISON OF NUMBERS OF MALE MRB CAUGHT IN PHEROMONE 1C STICKY TRAPS BAITED WITH PHEROMONE FORMULATED IN VARIOUS SUBSTRATES

Substrate[a]	Average moths caught/trap/night	Frequency/capture >0 (%)
Virgin females (3)	4.8 a[b]	92.1
Rubber septum	4.9 a	94.6
PVC rod	3.2 b	81.2
Plastic vial	1.7 bc	76.3
Hercon I	1.2 c	61.1
Hercon II	1.1 c	66.3

[a] All lures were prepared to contain 5.0 mg of MRB pheromone components in a ratio of 8:1:1.3 of (Z)-13-octadecenyl acetate, (Z)-11-hexadecenyl acetate, and (Z)-13-octadecenal, respectively.
[b] Means followed by the same letter are not significantly different at $P = 0.05$, Duncan's multiple-range test.

significantly fewer moths than traps baited with either three virgin females or pheromone-impregnated rubber septa, with approximately 65% as many captured in the PVC formulation. Traps baited with plastic vials or either of the two Hercon formulations captured ca. 20–30% as many male MRB as did virgin females or rubber septa impregnated with pheromone.

In other tests using the same substrates but varying the concentration of pheromone from 2.5 mg/lure to 10 mg/lure, relative catch was not improved in any of the substrates by either increasing or decreasing pheromone dosage. For example, 2.5, 5.0, and 10.0 mg of pheromone in rubber septa captured 8.3, 8.6, and 8.4 male MRB/trap/night, respectively, while the same three concentrations captured 5.4, 5.6, and 5.6 male MRB/trap/night, respectively, when formulated in black PVC rods. Although it is quite possible that other adjustments in component ratios or concentrations might have improved the relative performance of the plastic vial, black PVC, or the two laminated plastic lures, the pheromone formulated in rubber septa performed as well as three virgin females, so the rubber septum formulation was chosen for further testing.

Comparison of Pheromone Concentration. In the initial test (test 1) comparing concentrations of pheromone (8 : 1 : 1.3 ratio of Z-13-ODAc, Z-11-HDAc, and Z-13-ODAl, respectively) impregnated in rubber septa, no differences were observed in trap captures in any of the six dosages of 0.63–10.0 mg/lure (Table 2). Since there were no differences in captures during the early part of this test, a second test (test 2) was initiated one month later with these same six dosages but also included the two lower dosages of 0.15 and 0.31 mg/lure. During the first 44 days of the second test, no differences were observed in any of the eight dosages (Table 2). However, during the last 68 days of the test period, differences were noted between the lower two doses (0.15 and 0.31 mg) and the higher six doses (0.63, 1.25, 2.5, 5.0, 7.5, and 10.0 mg), with the higher doses capturing significantly more insects that lower ones.

Efficacy of the rubber septum formulation was confirmed by comparing the numbers of MRB caught in traps baited with the 1.25-mg and 5.0-mg lures that had been exposed to field conditions for 112 days in test 2 with catches by the 1.25-mg and 5.0-mg lures that had been prepared in the same batch but that had been stored tightly wrapped in aluminum foil in a freezer. During the 10 days of this comparison, no differences ($P = 0.05$, Duncan's multiple-range test) in trap capture were observed between exposed and unexposed lures, with captures of 9.2, 9.5, 12.4, and 10.1 moths/trap/night for the 1.25-mg lures exposed for 112 days or unexposed, respectively.

Comparison of Pheromone Component Ratios. When the various ratios of the three components (4 : 1 : 1.3 to 60 : 1 : 1.3) were compared in sugarcane fields in Universal bucket traps, the traps baited with the lower two ratios (4 : 1 : 1.3 and 8 : 1 : 1.3) captured the greatest numbers of male MRB (Table 3). Traps

TABLE 2. COMPARISON OF NUMBERS OF MRB CAUGHT IN INTERNATIONAL PHEROMONE SYSTEM BUCKET TRAPS BAITED WITH RUBBER SEPTA IMPREGNATED WITH MRB PHEROMONE WITH VARYING CONCENTRATIONS

Pheromone[a] concentration (mg/rubber septum)	Average moths caught (males/trap/night)[b]		
	Test 1	Test 2	
		1–44 days	45–112 days
0.15	3[c]	2.9a	8.9b
0.31	3[c]	3.3a	9.2b
0.63	10.1a	3.4a	16.4a
1.25	10.3a	3.5a	16.7a
2.5	11.4a	4.1a	15.2a
5.0	11.7a	3.8a	15.6a
7.5	11.3a	3.4a	14.9a
10.0	12.2a	4.0a	16.4a

[a] All lures were prepared to contain MRB pheromone components in a ratio of 8:1:1.3 of (Z)-13-octadecenyl acetate, (Z)-11-hexadecenyl acetate, and (Z)-13-octadecenal, respectively.
[b] Means followed by the same letter are not significantly different at $P = 0.05$, Duncan's multiple-range test.
[c] Not tested.

TABLE 3. COMPARISON OF NUMBERS OF NATIVE MRB MALES CAUGHT IN TRAPS BAITED WITH RUBBER SEPTA IMPREGNATED WITH 5 DIFFERENT RATIOS OF PHEROMONE COMPONENTS

Component ratio[a]	Average moths caught (males/trap/night)
4:1:1.3	7.8a[b]
8:1:1.3	6.6a
12:1:1.3	4.5b
36:1:1.3	1.5c
60:1:1.3	0.7c

[a] Component ratio refers to the ratio of (Z)-13-octadecenyl acetate, (Z)-11-hexadecenyl acetate, and (Z)-13-octadecenal, respectively. All lures contained 5 mg total pheromone.
[b] Means followed by the same letter are not significantly different at $P = 0.05$, Duncan's multiple-range test.

baited with the 12:1:1.3 formulation captured significantly more males than traps using either the 36:1:1.3 or 60:1:1.3 formulations. This indicates that as long as the component ratios are kept reasonably similar to 8:1:1.3, which was comparable to the ratio measured in ovipositor extracts of virgin MRB

females, efficacy as bait in MRB traps will be stable. However, if the relative proportion of Z-13-ODAc is increased much above 8 parts in the component ratios of 8 : 1 : 1.3, a significant decrease in MRB catches is expected.

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CINNAMYL DERIVATIVES AND MONOTERPENOIDS AS NONSPECIFIC OVIPOSITIONAL DETERRENTS OF THE ONION FLY

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Abstract—Laboratory dose–response choice tests and discriminate-dosage bioassays revealed wide variation in the effectiveness of cinnamyl, cinnamoyl, monoterpene, and phenethyl alcohol derivatives as ovipositional deterrents to *Delia antiqua* (Meigen), the onion fly. (*E*)-Cinnamic acids were not detectably deterrent. When formulated in particles of polyethylene glycol, (*E*)-cinnamaldehyde had a BR_{90} (concentration eliciting 90% deterrency) of 1.0% and (*E*)-4-methoxycinnamaldehyde had a BR_{90} of 0.38%. Among nine monoterpenoids tested, *p*-cymene was inactive, citronellal had a BR_{90} of 3.7%, and terpinene-4-ol had a BR_{90} of 0.46%. Para-substituted phenethyl alcohols gave increasing deterrence in the order: $-NO_2$, CH_3O-, $-Cl$, $-CH_3$, $-H$. Wide varieties of structures were deterrent: C-8 to C-13, intermediate in polarity, and possessing either oxygen-containing or nitrile functional groups. The air concentration of (*E*)-cinnamaldehyde at its BR_{90} was 1.7 ng/ml. This relatively high concentration, the diversity in deterrent structures, and the lack of differences in deterrency among positional and optical isomers suggest that ovipositional deterrency in onion flies is mediated by receptors broadly tuned for detecting phenylpropenoid, phenolic, monoterpenoid, and perhaps other classes of allelochemicals.

Key Words—Cinnamaldehyde, *Delia antiqua*, deterrent, discriminate-dosage bioassay, monoterpenoid, oviposition.

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INTRODUCTION

The onion fly, *Delia antiqua* (Meigen), is a serious pest of onions in northern temperate and Mediterranean climates worldwide (Loosjes, 1976). Alternative controls are needed for this pest, as it is increasingly difficult to control with conventional insecticides because of resistance development (Howitt, 1958; C.R. Harris et al., 1982) and because diurnal movement out of fields coincides with the usual timing for spray applications (Finch et al., 1986). Manipulation of ovipositional behavior might be effective in reducing pest pressure on highly susceptible seedlings.

Host-plant acceptance by insects usually involves both stimulatory and inhibitory external cues detected by various sensory modalities, integrated with internal physiological status (Dethier, 1982; Miller and Strickler, 1984). Both specialist and generalist phytophagous insects are thought to be sensitive to inhibitory stimuli. Therefore, deterrent chemicals have the potential to disrupt feeding and egg-laying, even on optimal host plants (Jermy, 1966; Jermy and Szentesi, 1978; Klocke et al., 1989).

Onion fly oviposition is stimulated by yellow or green vertical cylinders ca. 4 mm diameter that emit *n*-propyl disulfide from surface waxes (Harris and Miller, 1982, 1983, 1984). Surrogate onions with these characteristics elicit typical *D. antiqua* ovipositional behaviors, including foliar runs, ovipositor examining of foliage and soil, ovipositor probing, and egg deposition in the soil near the soil–foliage interface (Harris et al., 1987; Mowry et al., 1989). Surrogate onions provide the highly standardized ovipositional resource required for accurately testing putative ovipositional deterrents (Cowles et al., 1989).

Previous work with ovipositional deterrents for *Delia* flies has largely focused on mixtures of compounds from essential oils or other plant products. Havukkala (1982) had little success in field trials using turpentine-soaked stakes to control *D. radicum*, while Den Ouden and Theunissen (1980) achieved control of *D. brassicae* approaching standard chemical treatment in trials using slow-release formulations containing 2–8 g naphthalene per plant. Wiens et al. (1978) reported that hydrated bean seed extracts reduced onion fly oviposition by ca. 78% over controls in choice tests. The active constituent was a nonvolatile gustatory deterrent and was not further characterized. Alfaro et al. (1981) found that high levels (300 μg per ovipositional site) of red cedar leaf oil deterred *D. antiqua* oviposition by 84%. This activity was attributed to thujones, the major monooxygenated monoterpenoid components of this oil. Pine oil, a mixture of terpenes and oxygenated terpenoids, significantly reduced onion fly oviposition in a no-choice situation when 5 mg was applied per onion bulb (Javer et al., 1987).

Cowles et al. (1989) showed that black pepper, ginger, dill, and various materials containing red pepper deterred onion fly oviposition. Synthetic cap-

saicin, an analog of the principal flavor ingredient of red pepper, deterred oviposition by 95% when present at 320 ppm in the top cm of sand. Red pepper's efficacy disappeared in no-choice conditions, however, and was not effective in field tests.

In this study, we quantify the ovipositional deterrency of a range of cinnamyl, cinnamoyl, monoterpenoid, and phenethyl alcohol derivatives presented around surrogate onions exposed to *D. antiqua* in the laboratory. The goal was to explore structure–activity relationships and to target those compounds most promising for field assays.

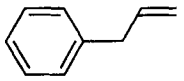
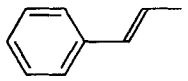
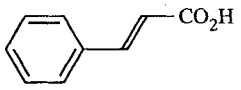
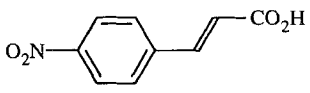
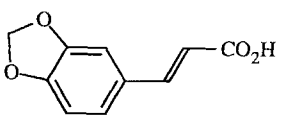
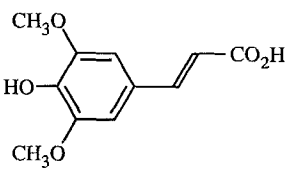
METHODS AND MATERIALS

D. antiqua *Culture*. A laboratory culture was started in September 1987 from pupae collected from onions left in harvested fields in Grant, Michigan (Newaygo County). Adults were housed in 80 × 65 × 60-cm screened cages provisioned with food (Schneider et al., 1983), water, and ovipositional resources (Harris et al., 1987). The rearing room was held at 16:8 light–dark photoperiod, 21 ± 1°C, and 70 ± 5% relative humidity. Larvae were reared on bisected onions. Flies used in all experiments were reared in the lab for 3–10 generations.

Chemicals. Most cinnamyl, cinnamoyl, and phenethyl derivatives were purchased from Aldrich Chemical Co. (Milwaukee, Wisconsin, 53233) or synthesized (Szurdoki et al., in preparation) in the Plant Protection Institute (Budapest, Hungary). The first set of the substances was simple derivatives of the parent compound **12** (Table 1), e.g., **54** and **20** (Reddy and Rao, 1980). Commercial (*E*)-cinnamaldehyde (**12**) served as starting material for the preparation of compounds **9, 11, 18,** and **34** (Allen and Edens, 1955, Kingsbury et al., 1976; Kowalski et al., 1982) and **13** (Varma and Kabalka, 1985) according to published methods. Afterwards, syntheses of several aldehydes with the formula $Ph(CH_2)_nCH{=}CH(CH_2)_mCHO$, where n, m = 0, 1, 2, 3, were performed. Thus (*E*)-α, β-unsaturated acid (I, Figure 1) (Gastaminza et al., 1984; Villiéras et al., 1985) was reduced to the corresponding allylic alcohol (II, e.g., **48**) (Ishizumi et al., 1968), which, in turn, was oxidized to the (*E*)-α, β-unsaturated aldehyde (III, m = 0, **41**) (Fatiadi, 1976). The bromo compound (**38**) obtained from (*E*)-cinnamyl alcohol (**13**), was transformed to $PhCH{=}CH(CH_2)_2CHO$ (**60**) via a malonester route. Subsequent Wittig coupling of 5-hydroxypentanal (Kienzle, 1980; Bestmann and Li, 1981) and oxidation of the alcohol product gave the aldehyde $PhCH{=}CH(CH_2)_3CHO$ (**44**; E/Z = 8:2).

Ferulonitrile (**46**) was used for the preparation of related derivatives **37** and **50**. Routine methods furnished compounds **31, 42, 53**, and **56**. Cinnamal-

TABLE 1. ESTIMATED MEAN AND 95% CONFIDENCE LIMITS OF BR_{90} OVIPOSITIONAL DETERRENCY OF CINNAMALDEHYDE DERIVATIVES

Compound	bp/pres. [°C]/[mm] (mp)[°C]	Source,[a] purity (%)	Structure	% of compound in PEG	
				Mean BR_{90}	95% CI
1 Allylbenzene	156	A, 98		Not active	
2 (*E*)-β-Methylstyrene	175	A, 99		Not active	
3 (*E*)-Cinnamic acid	300	A, 99+		Not active	
4 (*E*)-4-Nitrocinnamic acid	(285–286) dec.	H, 93		Not active	
5 (*E*)-3,4-Methylenedioxycinnamic acid	(242–243) dec.	H, 97		Not active	
6 Sinapinic acid 3,5-dimethoxy-4-hydroxycinnamic acid [predominantly (*E*)]	(203–205) dec.	A, 98		Not active	

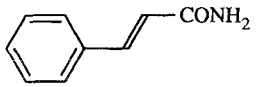
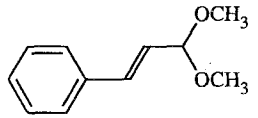
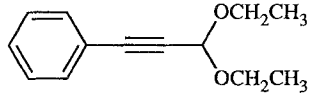
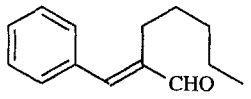
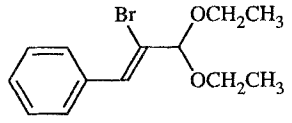
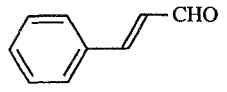
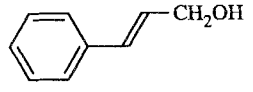
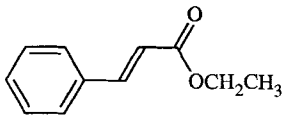
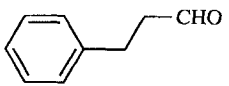
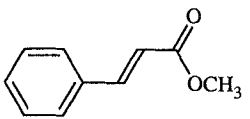
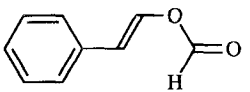
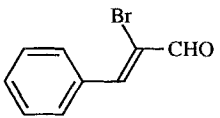
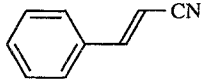
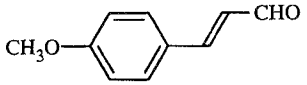
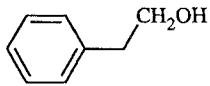
7 Cinnamamide [predominantly (*E*)]	(147)	A, 97		Not active	
8 (*E*)-Cinnamaldehyde dimethyl acetal	257[b]	I, 90		3.0	1.3–5.0
9 Phenylpropargyl aldehyde diethyl acetal	110–115/15	H, 95		2.5	0.92–5.6
10 α-Pentylcinnamaldehyde	287	A, 97		2.6	0.99–4.8
11 (*Z*)-α-Bromocinnamaldehyde diethyl acetal	145–150/5	H, 95		2.1	0.91–4.2
12 (*E*)-Cinnamaldehyde	248	A, 99		1.0	0.60–2.8
13 (*E*)-Cinnamyl alcohol	250	A, 98		0.88	0.60–1.8

TABLE 1. Continued.

Compound	bp/pres. [°C]/[mm] (mp)[°C]	Source,[a] purity (%)	Structure	% of compound in PEG	
				Mean BR_{90}	95% CI
14 Ethyl cinnamate [predominantly (*E*)]	271	A, 99		0.80	0.50–1.4
15 Hydrocinnamaldehyde	224	A, 95		0.84	0.50–1.2
16 (*E*)-Methyl cinnamate	260	A, 99		0.70	0.35–0.96
17 2-Phenylvinyl formate		N, 41		0.50	0.13–0.70
18 (*Z*)-α-Bromocinnamaldehyde	(67–68)	H, 98		0.44	0.22–0.64

19 Cinnamonitrile [predominantly (*E*)]	255	A, 97		0.43	0.15–0.74
20 (*E*)-4-Methoxycinnamaldehyde	(59–61)	H, 95		0.38	0.16–0.60
21 Phenethyl alcohol	220	A, 99		0.32	0.12–0.54

[a] Chemical sources: A, Aldrich Chemical, Milwaukee, Wisconsin, 53233; H, Plant Protection Institute, Budapest, Hungary; I, International Flavors and Fragrances, Inc., New York, New York; N, Dr. M.G. Nair, Horticulture Department, Michigan State University, East Lansing, Michigan.
[b] Boiling point corrected to 760 mm.

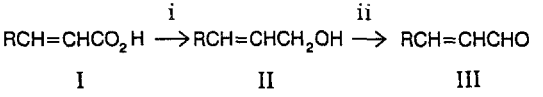


FIG. 1. Synthesis of α, β-unsaturated aldehydes. R: $Ph(CH_2)_n$, $n = 1, 2, 3$, or aromatic; double bonds have *E* geometry; i: 1. $ClCO_2Et$, EtN_3, THF 2. $NaBH_4$, H_2O; ii: MnO_2.

dehydes (Figure 1, III, R: aromatic), substituted at the benzene ring (e.g., **65**) were generated as depicted in Figure 1. Cinnamic esters (e.g., **51**) were prepared from the corresponding acids (Figure 1, I, e.g., compound **5**, obtained according to the Knoevagel-Doebner protocol) using established procedures.

Each compound was diluted in polyethylene glycol (PEG) (Carbowax PEG 8000, Fisher Scientific, Fair Lawn, New Jersey 07410) in five decade steps to a 0.001% concentration (w/w). PEG made an appropriate matrix for formulating these compounds as its polarity allowed compounds to dissolve readily and retarded evaporation. Preweighed PEG was melted at ca. 56°C, a candidate compound was added, and the sample was vortexed. The mixture was dispensed from a Pasteur pipet onto aluminum foil to form ca. 10-mg pellets, which were easily weighed for subsequent dilution steps. These pellets were coarsely crushed, then stored at −18°C until bioassayed. Experiments used 20 mg formulated mixture per treatment.

Bioassays. Ovipositional assays were conducted in a walk-in environmental growth chamber having conditions identical to the rearing chamber except that the bioassay room was free of onion volatiles. Experiments were conducted in 30-cm-diameter × 30-cm cylindrical cages provisioned with diet and water and stocked with ca. 10 male and 20 female flies. These cages rotated on their axis once every 13 min; thus, experimental error was minimized because environmental gradients were distributed evenly over all treatments (Weston and Miller, 1985).

Each ovipositional dish consisted of 50 g white silica sand (Unimin Granusil, Grade 40, Oregon, Illinois) moistened with 3 ml distilled water, then tamped into a 4-cm-diameter × 4-cm plastic cup. Standing upright in the center of each dish was a surrogate onion leaf consisting of a 0.4-cm-diameter × 12-cm glass tube painted onion-green with oil pigments (Winsor and Newton Cadmium Yellow, Flake Everwhite No. 2, Winsor Green, and Lamp Black in the weight ratio 132:7:5:2) and coated with paraffin wax containing 0.05% *n*-propyl disulfide (Harris et al., 1987). The surrogate onion was swiveled in the sand to provide a 1-mm space around its base for ovipositor probing (Mowry et al., 1989). This surrogate onion foliage is competitive with similarly sized onion foliage (Harris et al., 1987) and offers a highly controlled ovipositional resource. Deterrents were evenly dispensed on the sand within 1 cm of the

juncture of foliage and soil, where the most critical host examining behaviors and oviposition occur (Harris et al., 1987). PEG granules applied on moist sand partly dissolved and became incorporated in the top 1-mm layer.

For initial screening, the deterrency (percent reduction in the number of eggs laid on a treatment vs. the control) of ca. 1 mg of neat compound was compared with an untreated control. Four treatments were tested simultaneously per cage. If there was at least 80% deterrency in the first replicate, the compound progressed to dose–response analysis. If there was less than 80% deterrency recorded after three replicates, the BR_{90} (concentration required to elicit 90% behavioral response) was considered unattainable and the compound was listed as inactive.

Dose–response choice tests were conducted in a randomized complete block design. A replicate consisted of egg counts from six ovipositional resources (five decade step dilutions of a compound in PEG, plus a PEG control) placed 5 cm from the perimeter of the cage in randomized order and blocked by day. Five compounds were tested each day (one chemical in each of five cages), and flies were used once per compound; this ensured reasonable replicate independence and minimal fly learning. Replicates with combined treatment counts of less than 50 eggs were discarded to minimize sampling error. Every concentration series was replicated 10 times. Phenethyl alcohol was tested separately from the other compounds listed in Table 1. It was tested two days apart; all five cages were simultaneously used to obtain the 10 replicates.

Egg counts were not distributed normally; however, log-transformed [ln $(x + 1)$] counts fulfilled homogeneity of variance assumptions for analysis of variance (Bartlett's test, $P > 0.1$; excluding treatments with all zero egg counts and therefore zero variances). A two-way analysis of variance (general linear models procedure; SAS Institute, 1985) generated the treatment variance (mean square error). Ninety-five percent confidence limits were calculated for percent eggs relative to the control for each concentration in the dosage–response relationship for a compound. A procedure analogous to calculating the least significant ratio (LSR) (Steel and Torrie, 1980) was used, except that the confidence limit values $\{\text{mean} \,*/\div\, e[t_{\alpha/2}\sqrt{(MSE/r)}]\}$ were calculated. By connecting the upper limits and similarly connecting the lower limits, a 95% confidence belt was constructed for each dose–response relationship. The intersection of the confidence belt with the 90% deterrency response graphically estimated the concentration of chemical eliciting 90% ovipositional deterrency (BR_{90}) for a compound (Figure 2). This graphical method was used instead of regression analysis because dose–response functions were nonlinear and there were few deterrent concentration levels; therefore the regression degrees of freedom were low and confidence limits inflated. Furthermore, the graphical method makes better use of the knowledge that at high concentrations behavioral response converged

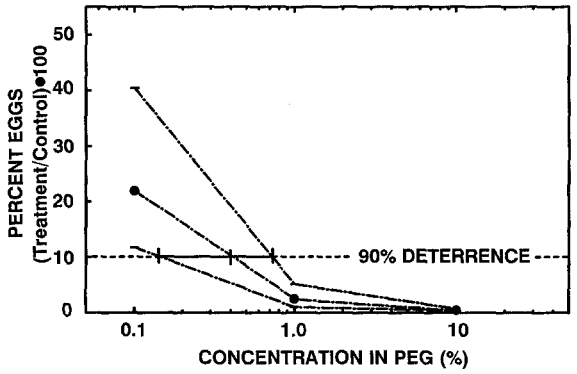


FIG. 2. Method for determining the BR_{90} from the dose–response relationship, as demonstrated with cinnamonitrile (see text for further explanation).

towards absolute deterrency, generating zero variance. Differences between apparently stimulatory responses of compounds at low concentrations and the control treatment were compared via the Student-Newman-Keuls' test.

Upon establishing that only the most active compounds at 0.1% were detectably different from control PEG, this concentration was chosen for conducting discriminate-dosage bioassays. In one large experiment, the activity of 35 additional compounds was ranked. Four randomly chosen materials and two blank PEG controls were placed in each of five rotating cages; cumulative presentation of all compounds constituted one replicate. Thirty female and 15 male flies were used for each of the four replicates of this modified randomized complete block design. Data were analyzed under the assumption that the number of eggs laid on each treatment relative to the number laid on the control is independent of the presence of other treatments (justified by unpublished data, Cowles). This allowed direct comparison of each compound to the control via a paired t test. When these data were log $(x + 1)$ transformed, the value used to test the null hypothesis, [TRT-(CON1 + CON2)/2] (SAS Institute, 1985), was a convenient transformation of percent acceptance, (TRT/CON) * 100%, since subtraction with logarithms is equivalent to forming this ratio. One slight difference is that averaging the two blank PEG controls in the former equation is equivalent to taking the geometric mean of the nontransformed counts. Using the difference between log-transformed egg counts satisfactorily transforms, and is symmetrical for, both deterrent (negative values) and stimulatory treatments (positive values). These data were then transformed to percent deterrency (or stimulation) (Table 3 below).

Vapor Concentration Required for Deterrency. Ovipositional cups were prepared with 20 mg of 1% (w/w) (*E*)-cinnamaldehyde or (*E*)-cinnamonitrile

in PEG placed on moistened sand, but without an onion foliar surrogate. One cup was placed in each of four cylindrical cages (the same as used for bioassays) and allowed to equilibrate in the 21°C conditions for 1 hr. A 1-ml air sample was withdrawn over a 45-sec interval with a Hamilton gas-tight syringe needle positioned within 5 mm of the PEG granules. The syringe needle was immediately inserted into a gas chromatograph injector port. The syringe was heated for 30 sec with a hand-held hair dryer to assure that the vapors did not condense on the syringe wall, then the plunger was immediately depressed to inject the sample. The Varian 3700 gas chromatograph was equipped with a 30-m × 0.32-mm-ID DB-5 capillary column. Running conditions were 120°C isothermal, 1 ml/min He carrier gas flow rate, with a flame ionization detector and HP3390A integrator. Standard curves were generated by injecting 1, 2, or 3 ng of each of the two test compounds in 1–2 μl of CS_2 (three replicates).

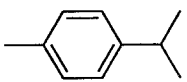
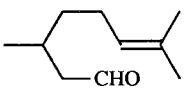
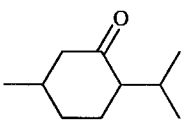
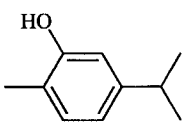
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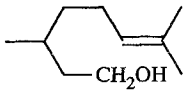
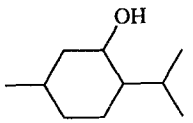
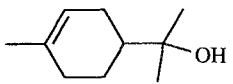
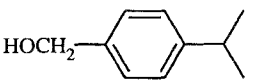
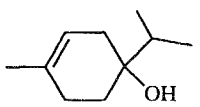
Dose–Response Series. Among cinnamyl-related compounds (Table 1), allyl benzene (**1**), (*E*)-β-methyl styrene (**2**), (*E*)-cinnamic acids (**3–6**), and (*E*)-cinnamamide (**7**) were less than 80% deterrent in the initial screening, so the BR_{90} was not attained. The mean BR_{90}s for active compounds ranged from 0.38% in PEG for (*E*)-4-methoxycinnamaldehyde (**20**) to 3% for (*E*)-cinnamaldehyde dimethyl acetal (**8**).

Ovipositional deterrent activity among terpenoids paralleled that of cinnamyl derivatives with similar functional groups (Table 2). The nonoxygenated monoterpene *p*-cymene (**22**), having the same carbon skeleton as terpenoids **24, 25**, and **27–30** in Table 2, was inactive in preliminary screening. The remaining oxygenated compounds had overlapping 95% confidence limits for their BR_{90}s, so their *D. antiqua* ovipositional deterrency did not differ statistically from each other.

Discriminate-Dosage Tests. Compounds tested at 0.1% concentration are ranked by increasing deterrency in Table 3. Because compounds were relatively dilute and variability was high, only the most deterrent compounds were significantly different from the control. Mean percent deterrency given in parentheses (compounds **31–37**) reveals instances when more eggs were laid on the chemical treatment than the control. Although none of these compounds was statistically different from the blank PEG, the 67% mean percent stimulation with benzalpinacolone (**31**) suggests that this compound is a likely ovipositional stimulant. Similarly, the close agreement of ca. 34% increase in numbers of eggs with 4-nitrophenethyl alcohol and (*E*)-4-nitrocinnamyl alcohol suggests that they are possible stimulants at 0.1%. Compounds **34–50**, with standard errors approaching or overlapping 0% deterrency, are probably neutral at the

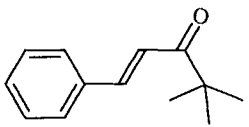
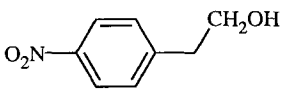
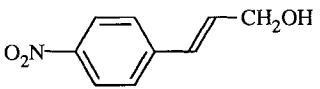
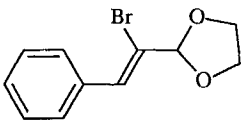
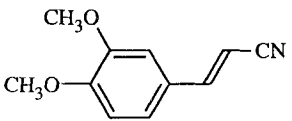
TABLE 2. ESTIMATED MEAN AND APPROXIMATE 95% CONFIDENCE LIMITS OF BR_{90} OVIPOSITIONAL DETERRENCY OF MONOTERPENOIDS

Compound	bp/pres. [°C]/[mm]	Source,[a] purity (%)	Structure	% of compound in PEG	
				Mean BR_{90}	95% CI
22 *p*-Cymene	176–178	A, 96		Not active	
23 Citronellal	207	S, 95		3.7	1.4 –5.8
24 Menthone (23% isomenthone)	210	A, 75		1.0	0.80–2.8
25 Carvacrol	236–237	A, 98		0.90	0.62–1.7

26 Citronellol	222	G, 92		0.88	0.72–1.2
27 Menthol	216	A, 99		0.86	0.64–1.0
28 α-Terpineol	217	A, 98		0.85	0.52–2.4
29 Cumic alcohol	135–136/26	A, 97		0.66	0.40–0.85
30 Terpinene-4-ol	210	A, 97		0.46	0.12–0.90

[a] Chemical sources: A, Aldrich Chemical, Milwaukee, Wisconsin, 53233; G, Givaudan Corp., Clifton, New Jersey, 07014; S, SCM Organic Chemicals, Jacksonville, Florida, 32201.

TABLE 3. ESTIMATED PERCENT OVIPOSITIONAL DETERRENCY (STIMULATION) FOR COMPOUNDS TESTED AT 0.1% CONCENTRATION IN PEG

Compound[a]	bp/pres. [°C]/[mm] (m.p.)[°C]	Source,[b] purity (%)	Structure	% Deterrency (Stim.) Mean	SE
31 Benzalpinacolone	(41–43)	H, 95		(67)	(87)–(16)
32 4-Nitrophenethyl alcohol	177/16	A, 99		(37)	(50)–(21)
33 (*E*)-4-Nitrocinnamyl alcohol	(122–125)	H, 90		(31)	(47)–(10)
34 (*Z*)-α-Bromocinnamaldehyde ethylene acetal		H, 95		(26)	(42)–(5)
35 (*E*/*Z*)-3,4-Dimethoxycinnamonitrile		A, 97		(26)	(34)–(17)

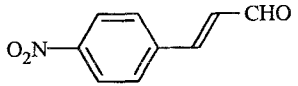
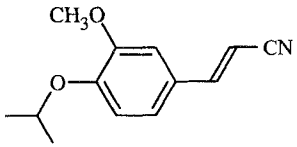
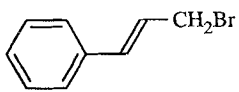
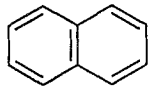
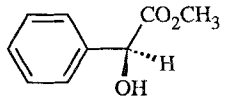
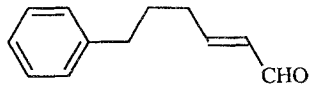
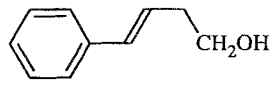
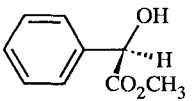
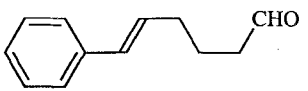
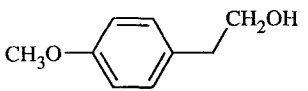
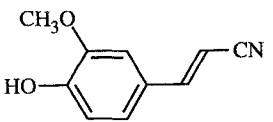
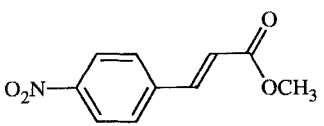
36 (*E*)-4-Nitrocinnamaldehyde	(139–141)	H, 95		(14)	(47)–28
37 (*E*/*Z*)-4-Isopropoxy-3-methoxycinnamonitrile (*E*/*Z* ~ 1 : 1)		H,95		(5)	(28)–20
38 (*E*)-Cinnamyl bromide	100–105/20	H, 90		4	(32)–38
39 Naphthalene		P, 99		6	(36)–44
40 (*S*)-(+)-Methyl mandelate	171	A, 99+		8	(53)–60
41 (*E*)-6-Phenyl-2-hexenal		H, 96		11	(14)–32
42 (*E*)-4-Phenyl-3-buten-1-ol		H, 95		21	(14)–46

TABLE 3. Continued.

Compound[a]	bp/pres. [°C]/[mm] (m.p.)[°C]	Source,[b] purity (%)	Structure	% Deterrency (Stim.) Mean	SE
43 (*R*)-(−)-Methyl mandelate	171[c]	A, 99+		21	(15)–48
44 (*E*/*Z*)-6-Phenyl-5-hexenal (*E*/*Z* ~ 8:2)		H, 90		22	(20)–51
45 4-Methoxyphenethyl alcohol	334	A, 99		23	(10)–47
46 Ferulonitrile (*E*/*Z* ~ 1:1)	160/1	H, 95		24	(22)–56
47 (*E*)-Methyl 4-nitrocinnamate	(158–161)	H, 90		30	(12)–44

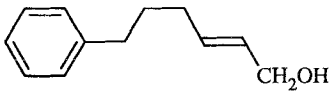
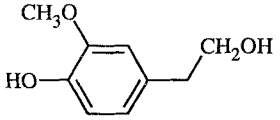
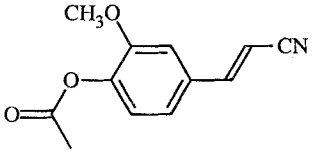
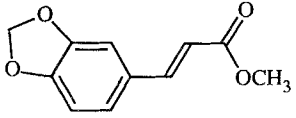
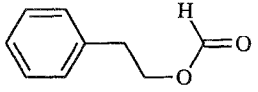
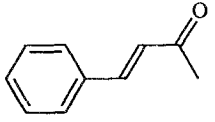
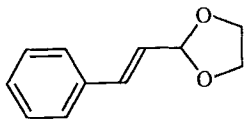
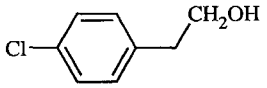
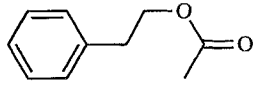
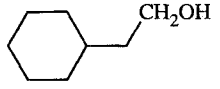
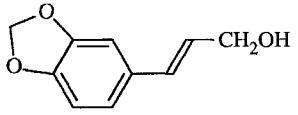
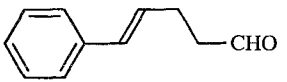
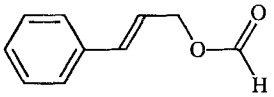
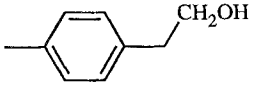
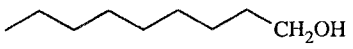
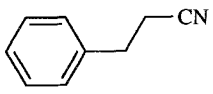
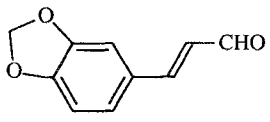
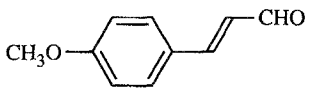
48 (*E*)-6-Phenyl-2-hexen-1-ol		H, 91		30	(10)–56
49 Homovanillyl alcohol		A, 99		31	1–52
50 (*E*/*Z*)-4-Acetoxy-3-methoxycinnamonitrile (*E*/*Z* ~ 1.4 : 1)		H, 95		32	4–51
51 (*E*)-Methyl 3,4-methylenedioxycinnamate	(131–133)	H, 93		48	9–70
52 β-Phenethyl formate		P, 95		50	18–70
53 Benzalacetone	(34–41)	H, 97		52	38–65

TABLE 3. Continued.

Compound[a]	bp/pres. [°C]/[mm] (m.p.)[°C]	Source,[b] purity (%)	Structure	% Deterrency (Stim.) Mean	SE
54 (*E*)-Cinnamaldehyde ethylene acetal		H, 95		58*[d]	43–69
55 4-Chlorophenethyl alcohol	310[c]	A, 99		59	21–79
56 Phenethyl acetate	232–235	H, 97		62*	45–73
57 2-Cyclohexylethanol	207	A, 99		63	13–84
58 3,4-Methylenedioxycinnamyl alcohol	(71–74)	H, 97		64*	64–71
59 Phenylpropargyladehyde	188/13	A, 96		69**	68–70

60 (*E*)-5-Phenyl-4-pentenal		H, 92		71*	60–79
61 Cinnamyl formate		P, 97		76*	74–77
62 4-Methylphenethyl alcohol	224	A, 99		78	50–90
63 1-Nonanol	215	A, 99		81*	68–88
64 Hydrocinnamonitrile	250[c]	A, 99		88*	78–93
65 (*E*)-3,4-Methylenedioxycinnamaldehyde	(84–86)	H, 95		88	70–95
20 (*E*)-4-Methoxycinnamaldehyde	(59–61)	H, 95		90**	86–92

[a] Cinnamaldehyde: 3-phenyl-2-propenal; cinnamic acid: 3-phenyl-2-propenoic acid; cinnamyl: 3-phenyl-2-propen-1-yl

[b] Chemical sources: A, Aldrich Chemical, Milwaukee, Wisconsin, 53233; H, Plant Protection Institute, Budapest, Hungary; P, Pfaltz and Bauer, Inc., Waterbury, Connecticut, 06708.

[c] Boiling point corrected to 760 mm.

[d] $*P < 0.05$, $**P < 0.001$; for t tests of $\ln(x + 1)$ treatment eggs versus controls, $N = 4$ for all compounds except **20**, $N = 20$ for **20**.

tested concentration. The remaining chemicals in the table were variably deterrent. Among those that were significantly different from the control, (*E*)-cinnamaldehyde ethylene acetal (**54**) was 58% deterrent (lowest) while hydrocinnamonitrile (**64**) was 88% deterrent (highest).

Vapor Concentration Required for Deterrency. The concentration of active compounds in air was quantified by gas chromatography. (*E*)-Cinnamaldehyde at its BR_{90} (1% formulation in PEG) was present at 1.7 ± 0.3 ng (mean ± SE) per milliliter of air. (*E*)-Cinnamonitrile is more active than cinnamaldehyde, so the 1% formulation would give greater than 90% deterrency; its vapor concentration was measured at 2.3 ± 0.3 ng/ml.

DISCUSSION

Advantages of Discriminate-Dosage Bioassay. Besides being able to select compounds with high activity, the discriminate-dosage bioassay could be completed approximately eight times faster than a full dose–response series. Furthermore, being able to test a large number of compounds in one experiment permitted a larger number of compounds to be compared directly. The 90% deterrency estimate for (*E*)-4-methoxycinnamaldehyde (**20**), which was included as a positive control in half the discriminate-dosage replicates, agreed well with the estimated BR_{90} concentration of 0.4% from the dose–response assays.

A large amount of structure–activity information was obtained by combining data from dose–response and discriminate-dosage bioassays. Instead of using the BR_{90} values from Tables 1 and 2, deterrency observed at the 0.1% PEG concentration in dose–response assays was directly compared to that from the discriminate-dosage tests.

Structure–Activity Relationships. Key features investigated in this study were unsaturation and length of alkyl side chains, functional groups at the end of an alkyl side chain, ring substitution, and presence of an aromatic ring. Unsaturation and the accompanying conjugation between the aromatic ring and carbonyl in cinnamaldehyde was not necessary for activity. (*E*)-Cinnamaldehyde (**12**) and hydrocinnamaldehyde (**15**) had similar BR_{90}s of about 0.9% (Table 1) and had similar dose–response profiles. Hydrocinnamonitrile (**64**) and cinnamonitrile (**19**) had similar deterrency (ca. 85%) at the 0.1% concentrations. However, the alkyne bond in phenylpropargylaldehyde (**59**) and its diethyl acetal (**9**) appeared to enhance activity compared to the saturated or alkene compounds (**15, 12, 8**).

Increasing the length of the alkyl side chain of aromatic compounds appeared to decrease activity. (*E*)-5-Phenyl-4-pentenal (**60**) was deterrent while (*E*)-6-phenyl-5-hexenal (**44**) was not. An alkyl side chain also decreased activity of **10** vs. **12**. Phenethyl alcohol (**21**), included as a comparison to cinnamyl

alcohol (**13**), had the highest activity (BR_{90} of 0.32%), suggesting that a two-carbon side chain is no less active than a three-carbon side chain. Part of the loss in activity with alkyl group size probably is due to the attendant decrease in volatility. The vapor pressure of these compounds is inversely related to the boiling points given in the tables.

Moderately polar functional groups appear to be required for ovipositional deterrency. Hydrocarbons (1, 2, 22) were inactive at the extreme for nonpolar compounds. Carboxylic acids (**3–6**) and cinnamamide (**7**) are relatively polar compounds and also were inactive. Functional groups associated with deterrency were ketones, aldehydes, alcohols, nitriles, and esters. The contrast between aldehydes and alcohols (**12** vs. **13, 23** vs. **26**) seems to indicate that alcohols had greater activity.

Modifications of aldehydes included formation of their acetals and α-bromination. Acetals (**8, 11, 34**, and **54**) were investigated because they might allow slow chemical release (Pickett et al., 1984) of the parent aldehydes (**12, 18**). Acetalation reduced activity; this could be attributed to reduced fit to a receptor and/or decreased volatility. α-Bromo derivatives (**18, 11**) were more active than the nonhalogenated compounds (**12, 8**).

Ring substitution at the para position of phenethyl alcohol (**21, 32, 45, 55, 62**) decreased deterrency in the following order: unsubstituted, methyl, chloro, methoxy, nitro. Nitro-substitution converted deterrent phenethyl alcohol (**21**), cinnamaldehyde (**12**), and cinnamyl alcohol (**13**) into possibly oviposition-stimulating chemicals (**32, 36**, and **33**, respectively). Except for the 3,4-methylenedioxy-substituted cinnamaldehyde (**65**), multiple ring substitution seemed to reduce deterrency compared to unsubstituted or monosubstituted rings. For example, none of these compounds (**37, 46, 49, 50**, and **51**) were significantly deterrent in the discriminate assay. One half as much terpinene-4-ol (**30**) was required as carvacrol (**25**) to achieve 90% deterrency, suggesting that the OH ring position may affect deterrency.

The closely similar activity of carvacrol (**25**) and citronellol (**26**) suggests that the aromatic ring is not a key deterrent feature. This is confirmed by the deterrency of 1-nonanol (**63**).

Biological Activity as Influenced by Chemical Concentration. As summarized by Dethier (1947), many chemicals are deterrent or repellent at high concentrations, including host-plant chemicals that at lower concentrations mediate host acceptance. For example, *n*-propyl disulfide is deterrent at high concentrations (Matsumoto and Thorsteinson, 1968; Harris et al., 1987) and stimulatory at an optimum concentration in onion surface wax of about 0.05%. Demonstrating that these compounds were not simply deterrent because of elevated dosages was particularly important because phenethyl alcohol, the most active deterrent in these assays, had been described by other workers as an attractant for both onion and seed-corn flies (Ishikawa et al., 1983). The dose–response

choice tests were well suited to ascertain deterrent potency at a range of concentrations; the discriminate-dosage bioassays also address stimulation at low concentrations. Any material that is stimulatory at low concentrations would be problematic when applied in the field, because the concentration eventually would decrease to levels stimulating undesirable insect behaviors. None of the cinnamaldehyde or terpenoid derivatives tested in dose–response assays elicited increased oviposition through the range of rates tested, although, as mentioned above, some nitro-containing compounds assayed via discriminate-dosage may be somewhat stimulatory.

The concentration of compounds from both groups of compounds required to deter ovipositional activity and the threshold for detecting EAG response to these compounds (Cowles, unpublished data) indicate that onion flies have approximately a 10- to 100-fold greater sensitivity to the stimulatory compound, *n*-propyl disulfide, than to these deterrents. These generalizations agree well with Dethier's (1980) proposition that receptors sensitive to deterrents should be relatively less specialized, and therefore have higher sensitivity thresholds, than receptors that are adapted for detecting host-specific stimuli.

Behavioral Effects of These Deterrents and Volatility. At the highest dosages of deterrent compounds, flies were observed to move away from the source after approaching to within ca. 1 cm. Thus, they act as repellents. Part of the reason why some compounds in this study are more active than capsaicin-containing products (Cowles et al., 1989) is that deterrency could be mediated not only by short range gustation (as with capsaicin) but also through olfaction.

Deterrency is not a simple function of volatility, however. This is demonstrated with the hydrocarbons that are highly volatile but inactive and by the high deterrency of less volatile compounds, such as 4-methoxycinnamaldehyde (**20**) and α-bromocinnamaldehyde (**18**).

Suggested Mode of Action for D. antiqua *Ovipositional Deterrents.* Deterrents can be hypothesized to have various modes of action (Davis, 1985). They could: (1) block perception of host stimuli by binding to and inhibiting their receptors (Davis, 1985), (2) cause more permanent damage to chemoreception by cross-linking or otherwise disturbing receptor proteins (Gothilf and Bar-Zeev, 1972), (3) act upon specialized deterrent receptors responsive to nonhost odors or eidectic pheromones (Prokopy, 1981), or (4) be detected by generalized receptors and interpreted as deterrents by the CNS. The broad array of deterrent compounds, including monoterpenoid ketones and alcohols, substituted phenethyl alcohols, 1-nonanol, and cinnamyl-related aldehydes, their acetals, alcohols, and nitriles, is unsupportive of the idea that *D. antiqua* deterrency requires functional group specificity, and thus is unsupportive of hypotheses 1, 2, and 3 (above). Indeed, the similar deterrency ranking of positional (**41** and **44**) or optical (**40** and **43**) isomers argues against highly specific receptor geometry. The large variation in deterrent structure, both in size and functional moieties,

requires that *D. antiqua* possess either a very broadly tuned population of one chemoreceptor type, or more likely, a variety of receptor types that detect these compounds. For onion fly ovipositional deterrency, we favor hypothesis 4 (above), and recognize that dealing with multiple deterrent receptors complicates optimization of compound "fit" to receptors via structure–activity relationship analyses, particularly if stimulus inputs interact in the CNS.

Consideration of Stimulus Interaction. Host odors can act synergistically during host finding or acceptance (Finch, 1978; Visser, 1983; Davis and Sokolove, 1976), probably via central integration of signals from various receptors (Visser, 1983). At the ecological level, integrating signals from various receptors provides far greater information than relying on signals from one receptor type. Combining inputs, both within and across sensory modalities (Miller and Strickler, 1984) thus reduces uncertainty in the host acceptance decision-making process. Such a scheme also holds for interpreting compounds as deterrents, hence presentation of single deterrent compounds may not be the best way to maximize deterrence. Instead, synergistic combinations of compounds that act upon several receptor populations might be most deterrent.

Complex involvement of multiple receptor populations could help explain inconsistencies in the rank order for deterrency based on specific chemical modifications. For example, *para*-methoxy substitution (**20, 45**) increased activity for (*E*)-cinnamaldehyde (**12**), but not for phenethyl alcohol (**21**). Another example is the reduced activity of 3,4-methylenedioxycinnamyl alcohol compared to the aldehyde (**58** vs. **65**), which reverses the trend seen with other alcohol–aldehyde comparisons.

Ecological Interpretations. Why are onion flies deterred by such a broad array of chemicals? The phenylpropenoids, phenolics, and terpenoids tested in this study are well-known allelochemicals. In plants, they prevent competition by inhibiting root growth or seed germination (Rice, 1984; Nair et al., 1988), inhibit microbes (Levin, 1971; Mitscher, 1975; Mandava et al., 1980), and are toxic to or block detoxification enzymes in insects (Brattsten, 1983; Singh et al., 1989). These same compounds can be sequestered by or synthesized by insects for defense (Eisner, 1970; Rodriguez and Levin, 1976) and can act as cues for the presence of interspecific competitors (Jones et al., 1988). More complex multitrophic interactions (Howard et al., 1988) can be governed by plant defensive compounds. Indeed, the ability of herbivores or predators to sense these chemicals allows them to function as interspecific warning signals (Lovett et al., 1989). Such signals serve the evolutionary interests of both the sender, who benefits by deterring enemies, and the receiver, who benefits by avoiding a source of potentially toxic substances. For the insect not adapted to detoxify phenolics or terpenoids in their host plant or environment, these chemicals could, over evolutionary time, become grouped to indicate poor suitability.

Sensitivity to phenolics and terpenoids appears to be a general phenomenon in insects and suggests that chemoreceptors for these compounds might be ancient. Mosquito feeding repellents (Bunker and Hirschfelder, 1925) and ovipositional deterrents (Klocke et al., 1989) have structure–activity relationships very similar to those of the present study; activity is largely governed by oxygenation of C_7 to C_{13} compounds. Thus, the "oxygenated" derivatives of inactive hydrocarbons (aldehydes, esters, ketones, and alcohols) exhibited strong repellent activity. If terpenoids and phenylpropenoids are detected by the same receptors, then we would expect the magnitude of insect response (attraction or avoidance) to be correlated with fit of compounds to those receptors. In some cases, species are attracted to the same compounds that are deterrent to onion flies. For example, in trapping experiments using bait pails for codling moths, citronellal and methyl cinnamate have been shown to be attractants (Dethier, 1947). Specific structure–activity relationships have been elucidated for attraction of *Diabrotica* species to cinnamyl derivatives. Among these derivatives, *Diabrotica* species responded most strongly to 4-methoxycinnamaldehyde, 4-methoxycinnamonitrile, cinnamyl alcohol, and cinnamaldehyde (Metcalf and Lampman, 1989), the same compounds with greatest onion fly deterrency.

Although general sensitivity of insects to these compounds may be exploitable, specific ecological relationships are certain to fine tune the behavioral response and reorder the ranking of activity. For example, even though codling moths are sensitive to the same classes of compounds as are onion flies, their rank order for sensitivity to acids, esters, and alcohols (Dethier, 1947) is the reverse of what we observed for onion flies, probably because they are adapted to fruit feeding. Recently, Jones et al. (1988) discovered that 3,5-dimethoxy-4-hydroxycinnamic acid, produced in the frass of a lepidopteran pest of cabbage, deters oviposition by *D. radicum*, suggesting that this *Delia* species may have greater sensitivity to carboxylic acids than does the onion fly.

Practical Considerations. There are practical advantages and disadvantages to the finding that deterrents or repellents act upon fairly generalized chemoreceptors. On one hand, generality could allow a single product to be broadly useful in agriculture because of the wide range of pests that could be deterred. On the other hand, higher concentrations are required than for detecting host cues. Field studies with thymol, eugenol, cinnamaldehyde and its dimethyl acetal, phenethyl alcohol, and cinnamyl formate (Cowles, unpublished data) suggest that practical use of ovipositional deterrents will rely on placing these compounds in close enough proximity to a host plant and at sufficiently high concentrations. For the volatile compounds (*E*)-cinnamaldehyde and (*E*)-cinnamonitrile, the concentration of active material required for at least 90% deterrency appears to be 1–2 ng/ml of air, which is nine orders of magnitude more concentrated than for obtaining the BR_{50} for some lepidopteran

pheromones (Miller, unpublished data). Because of the high concentrations of these compounds needed for control, care will be required that formulations be designed to avoid phytotoxicity.

We believe that these ovipositional deterrents should be explored further as possible agents of pest control. It is not yet clear whether deployment would involve controlled release formulations of synthetics, companion plants chosen for release of these deterrents, or using plant breeding or genetic engineering to incorporate these agents into the crop plants. Whatever the deployment method, it will be important that onion flies are given highly attractive alternative sites in which to oviposit (i.e., stimulo-deterrent diversion; Miller and Cowles, 1990), so that pest deprival does not lead to loss of deterrency.

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EFFECTS OF FERULIC ACID, AN ALLELOPATHIC COMPOUND, ON NET P, K, AND WATER UPTAKE BY CUCUMBER SEEDLINGS IN A SPLIT-ROOT SYSTEM[1]

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Abstract—Since distribution of allelopathic compounds in soils is highly variable, injurious effects by such compounds should be related to the frequency of contact with roots. Experiments were conducted to determine how P, K, and water uptake of cucumber seedlings were affected as the fraction of roots in contact with ferulic acid (FA) was increased. Seedlings were grown in Hoagland's nutrient solution for 14 days and then transferred to 0.5 mM $CaSO_4$ solution for 24 hr before being placed into a split-root culture system. The containers in the system were filled with 0.5 mM concentrations of KH_2PO_4 and $CaSO_4$ or 0.5 mM concentrations of KH_2PO_4, $CaSO_4$, and ferulic acid (FA). Net uptake of P by seedlings (milligrams per seedling) decreased in a curvilinear (concave) manner as the fraction of the roots in contact with FA increased. Net uptake of K (milligrams per seedling) and water (milliliters per seedling) by seedlings decreased linearly as the fraction of the roots in contact with FA increased. Net uptake of P, K, and water by seedlings was reduced 57, 75, and 29%, respectively, when the whole root system was exposed to FA. Net P and K uptake of roots (milligrams per gram root fresh weight) not in contact with FA decreased in a linear and curvilinear (convex) manner, respectively, as the fraction of roots in contact with FA increased. Net P and K uptake of roots in contact with ferulic acid increased in a linear and curvilinear (convex) manner, respectively. Net water uptake of roots (milliliters per gram root fresh weight) not in contact with FA increased in a curvilinear (concave) manner as the frequency of the roots in contact with

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FA increased. Net water uptake of roots in contact with FA did not show a trend. Transpiration (milliliters per square centimeter) was reduced in a linear manner as the fraction of roots in contact with FA increased. A very slight compensation by roots not in contact with FA for roots in contact with FA was observed for net water uptake rates. No compensation for P and K uptake rates was observed.

Key Words—*Cucumis sativus*, ferulic acid, split root, phosphorus, potassium, water, net uptake.

INTRODUCTION

The distribution of allelopathic compounds in soils varies both horizontally and vertically (Patrick et al., 1964; Blum and Rice, 1969; Patrick, 1971; Dao, 1987). It is thus unlikely that whole root systems are subject to the impact of allelopathic compounds at any given point in time. This has led several researchers to suggest that injurious effects by allelopathic compounds should be related to the frequency of contact (Patrick, 1971; Blum and Rebbeck, 1989; Klein and Blum, 1990). Such a suggestion assumes that roots not in contact with allelopathic compounds do not compensate for roots in contact with allelopathic compounds. Klein and Blum (1990) noted that leaf expansion of cucumber seedlings was inversely related to the proportion of the root system in contact with ferulic acid (FA). Blum and Rebbeck (1989) noted that leaf area expansion and root growth of cucumber seedlings quickly recovered once FA was removed from the root environment.

FA is known to inhibit the uptake of phosphorus (P) and potassium (K) (Glass, 1975; McClure et al., 1978; Harper and Balke, 1981; Kobza and Einhellig, 1987; Mersie and Singh, 1988) and to reduce water utilization (Einhellig and Kuan, 1971; Einhellig and Muth, 1980; Blum et al., 1985; Einhellig et al., 1985). It also has been observed that at least some plants can adapt to localized sources of P by adjusting root growth and/or uptake (Drew, 1975; de Jager and Posno, 1979; de Jager, 1982). Whether such adjustments may occur when only a portion of a root system is in contact with FA is not known.

The present investigation was conducted to answer two questions: (1) How would the impact of FA on uptake of P, K, and water be modified as the fraction of the root system in contact with FA increased? and (2) could nontreated FA roots compensate in P, K, and water uptake for FA-treated roots?

METHODS AND MATERIALS

Plant Material. Seeds of *Cucumis sativus* cv Early Green Cluster (Wyatt Quarles Seed Company, Raleigh, North Carolina) were germinated for three days in the dark at 30°C in trays containing vermiculite and full-strength Hoag-

land's nutrient solution (Hoagland and Arnon, 1950). Germinated seeds were exposed to 13 hr of light and 11 hr of dark before transplanting them to 120-ml jars containing Hoagland's solution (Blum and Dalton, 1985). Seedlings were grown for 10 days under three light banks (150 $\mu E/m^2/sec$; 13-hr photoperiod) at room temperature (21–31°C). Nutrient solutions (pH 5.5) were changed on day 5. Nutrient solutions were replaced with 0.5 mM $CaSO_4$ solutions when seedlings were 14 days old. All solutions were continuously aerated.

Split-Root Experiments. On day 15, seedling roots were suspended in a 700-ml container that enclosed two smaller containers each filled with 100 ml of treatment solution (Klein and Blum, 1990). KH_2PO_4 was used as the phosphorus and potassium source for all treatment solutions, while $CaSO_4$ was added to maintain membrane function and metabolic regulation. All solutions were adjusted to a pH of 5.5 with NaOH or HCl. Treatment solutions were as follows: 0.5 mM KH_2PO_4 and $CaSO_4$ in both containers; 0.5 mM KH_2PO_4, $CaSO_4$, and FA in both containers; or one container with 0.5 mM KH_2PO_4 and $CaSO_4$ and the other container with 0.5 mM KH_2PO_4, $CaSO_4$, and FA. Roots were split between these two containers so that the fraction of roots treated with the 0.5 mM FA ranged from 0 to 1 by weight. Twenty-four hours after treatment, seedlings were harvested.

Measurements. Leaf area was determined before and after treatment (Blum and Dalton, 1985). Root and shoot fresh weight were determined at harvest. The volume of each solution remaining at harvest in each container was measured to determine water utilization. For each solution, content of phosphorus was determined colorimetrically (Taussky and Shorr, 1953) and content of K by atomic absorption spectrophotometry. Net uptake of P, K, and water was based on depletion of these components from the initial 100-ml solutions Transpiration was estimated by dividing total water depletion for a seedling by its mean 24-hr leaf area.

Data Analyses. Treatments ($N = 50$) were randomly distributed under three light banks. Analysis of variance and linear and multiple regression analyses (SAS, 1988) were used to determine differences and trends.

RESULTS

Preliminary Experiments. In a previous study, it was determined that detection of treatment effects on net uptake of P, K, and water required at least 8 hr when the whole root system was exposed to 0.5 mM FA in 100 ml of treatment solution (Lyu and Blum, unpublished data). After some additional experimentation, we concluded that 24 hr was required to detect changes in net uptake for the smaller root biomass in the split-root experiment.

Plant Response. Leaf area (62 $\pm$ 2 cm^2, control mean $\pm$ SD) and shoot fresh weight (3.17 $\pm$ 0.19 g) of cucumber seedlings were not significantly mod-

ified when the whole root system was treated with 0.5 mM FA for 24 hr. Root (1.33 ± 0.03 g) and plant (4.52 ± 0.21 g) fresh weights and absolute rates of leaf expansion (11.18 ± 1.92 cm^2/day) were reduced 23, 13, and 35%, respectively, for this treatment when compared to seedlings not treated with FA.

Phosphorus Uptake. The net uptake of P per seedling during the 24-hr treatment period decreased in a curvilinear manner (mg P/seedling = 0.713 − 0.934 (RFA) + 0.569 $(RFA)^2$, where RFA = fraction of roots in FA; r^2 = 0.52, P = 0.0001) as the fraction of the roots in contact with 0.5 mM FA increased (Figure 1). Net P uptake of seedlings given the whole-root FA treatment was reduced 57% when compared to the seedlings not treated with FA. Half the observed reduction occurred when approximately 23% of the root system was in contact with FA. Maximum inhibition of net P uptake occurred when 82% of the root system was in contact with FA.

Net P uptake of roots not in contact with FA [mg P/g root fresh weight (RFW)] decreased as the fraction of such roots decreased (Figure 2A). The slope of this regression line was −0.217 (r^2 = 0.26, P = 0.0006). Net P uptake of roots in contact with FA increased as the fraction of such roots increased (Figure 2B). The slope of this regression line was 0.229 (r^2 = 0.18, P = 0.0057).

Potassium Uptake. Net uptake of K per seedling during the 24-hr treatment period decreased in a linear manner (slope = −0.966, r^2 = 0.35, P = 0.0001) as the fraction of the roots in contact with 0.5 mM FA increased (Figure 3).

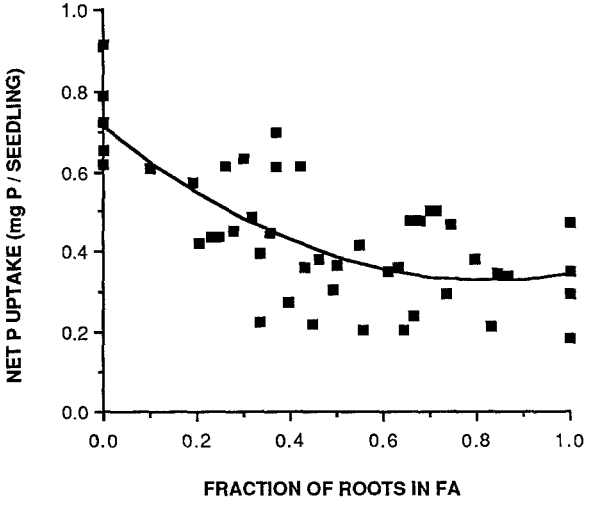


FIG. 1. Net uptake of phosphorus (P) by cucumber seedlings as the fraction of roots in contact with ferulic acid (FA) increased.

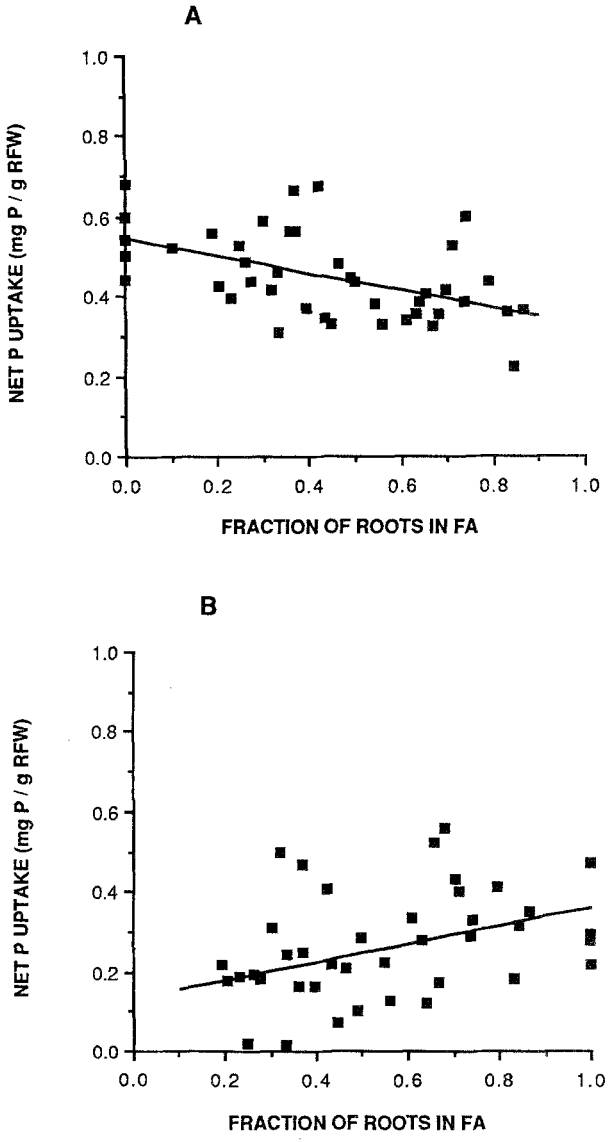


FIG. 2. Net uptake of phosphorus (P) by roots of cucumber seedlings (A) not in contact with ferulic acid and (B) in contact with ferulic acid as the fraction of roots in contact with ferulic acid (FA) increased.

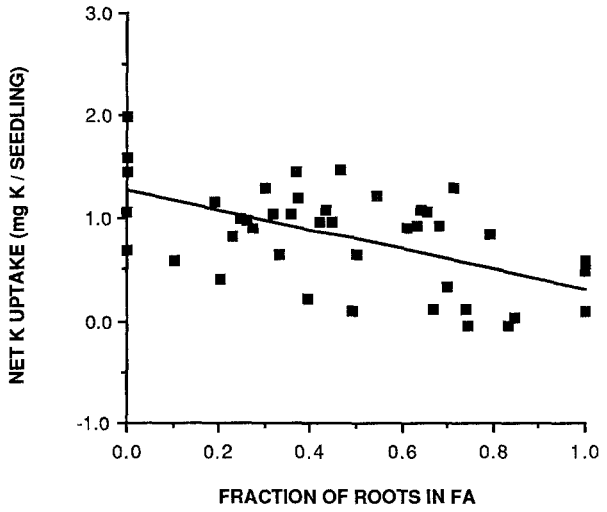


FIG. 3. Net uptake of potassium (K) by cucumber seedlings as the fraction of roots in contact with ferulic acid (FA) increased.

Net K uptake of seedlings given the whole-root FA treatment was reduced 75% when compared with the seedlings not treated with FA.

Net K uptake of roots not in contact with FA decreased in a curvilinear manner (mg K/g RFW = 0.894 + 1.826 (RFA) − 3.470 $(RFA)^2$, where RFA = fraction of roots in FA; $r^2 = 0.22$, $P = 0.011$) as the fraction of such roots decreased (Figure 4A). Net K uptake of roots in contact with FA increased and then decreased in a curvilinear manner (mg K/g RFW = −1.699 + 7.573 (RFA) − 5.760 $(RFA)^2$, where RFA = fraction of roots in FA; $r^2 = 0.25$, $P = 0.0055$) as the fraction of such roots increased (Figure 4B). Variation of net uptake, however, was considerable for the lower root fractions treated with and without FA.

Water Uptake. Net water uptake per seedling during the 24-hr treatment period (ml water/seedling) decreased in a linear manner (slope = −7.405, $r^2 = 0.19$, $P = 0.002$) as the fraction of the roots in contact with 0.5 mM FA increased (Figure 5). Net water uptake was reduced 29% for seedlings given the whole-root FA treatment when compared with seedlings not treated with FA.

Net water uptake of roots not in contact with FA increased in a curvilinear manner (ml water/g RFW = 25.251 − 6.803 (RFA) + 31.751 $(RFA)^2$, where RFA = fraction of roots in FA; $r^2 = 0.51$, $P = 0.0001$) as the fraction of such roots decreased (Figure 6). This was in contrast with the roots in FA for which no significant trends were noted.

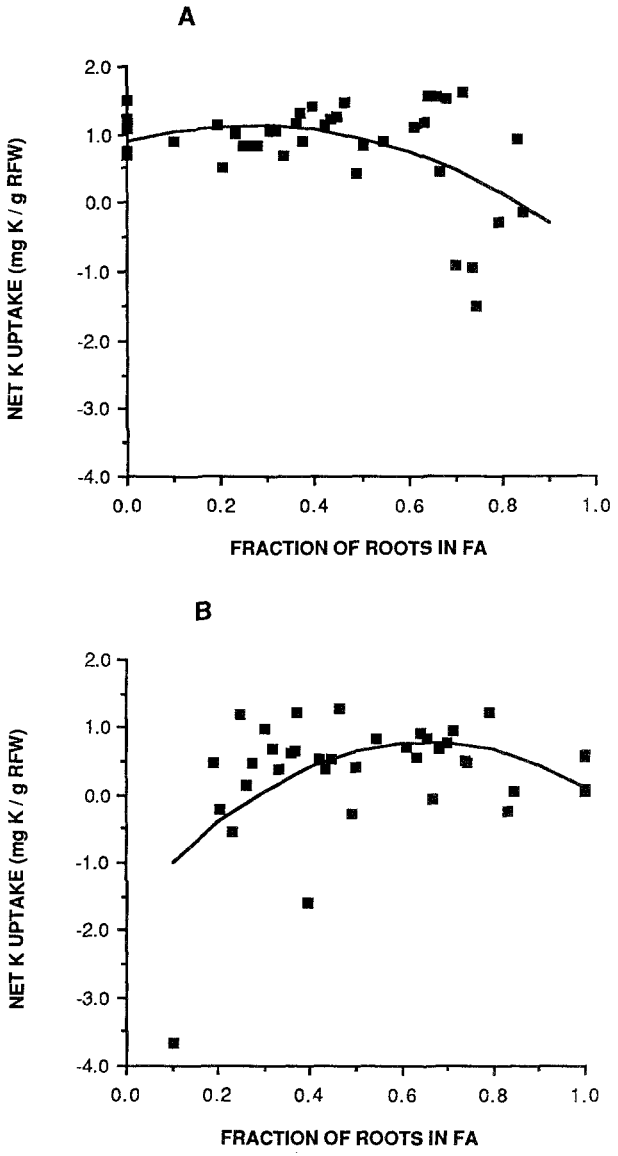


FIG. 4. Net uptake of potassium (K) by roots of cucumber seedlings (A) not in contact with ferulic acid and (B) in contact with ferulic acid as the fraction of roots in contact with ferulic acid (FA) increased.

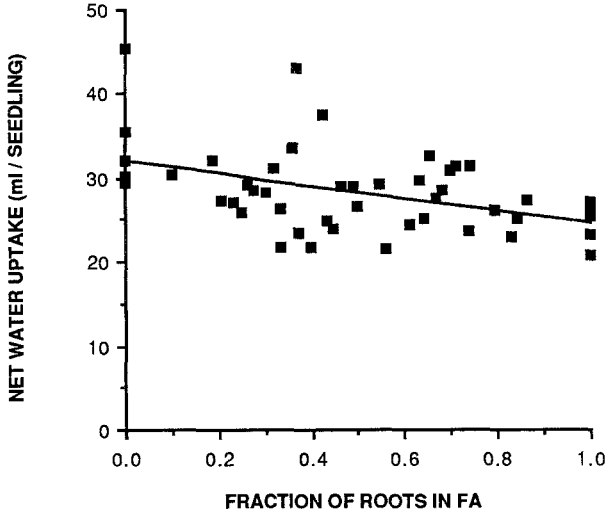


FIG. 5. Net water uptake by cucumber seedlings as the fraction of roots in contact with ferulic acid increased.

Transpiration (ml water/cm^2 leaf area) decreased in a linear manner (slope $= -0.086$, $r^2 = 0.095$, $P = 0.035$) as the fraction of roots in contact with FA increased (Figure 7). Transpiration rates were reduced 11% for seedlings given the whole-root FA treatment when compared to seedlings not treated with FA.

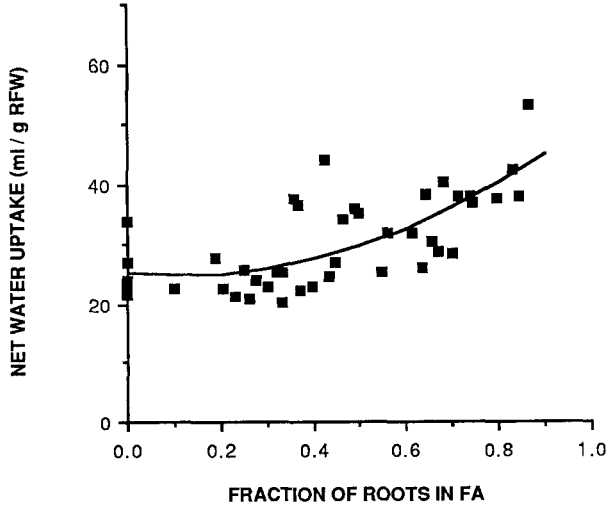


FIG. 6. Net water uptake by roots of cucumber seedlings not in contact with ferulic acid (FA) as the fraction of roots in contact with FA increased.

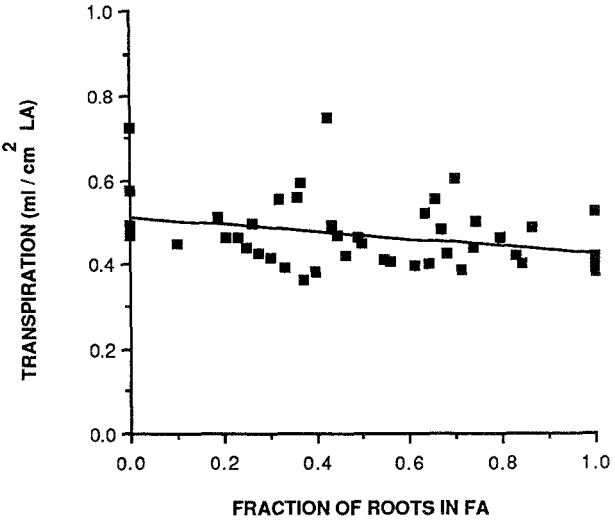


FIG. 7. Transpiration of cucumber seedlings as the fraction of roots in contact with ferulic acid (FA) increased. LA = leaf area.

DISCUSSION

The impact of ferulic acid (FA) on net uptake of P, K, and water by cucumber seedlings was related to the fraction of the roots in contact with FA. When the whole root system was exposed to 0.5 mM FA, seedling net uptake of P, K, and water was reduced 57, 75, and 29%, respectively, compared to seedlings not treated with FA. The observation that exposure of whole root systems or excised roots to FA result in an inhibition of net uptake of P, K, and water is not new, since this has been observed for a variety of species (Einhellig and Kuan, 1971; Glass, 1975; McClure et al., 1978; Einhellig and Muth, 1980; Harper and Balke, 1981; Blum et al., 1985; Einhellig et al., 1985; Kobza and Einhellig, 1987; Mersie and Singh, 1988). In addition, it has been shown that the impact of FA on cucumber seedling growth increases as the fraction of the roots in contact with FA increases (Klein and Blum, 1990). What is new is that for the first time it has been shown that the impact of an allelopathic phenolic acid on net uptake of P, K, and water is related to the fraction of the roots in contact with a phenolic acid. Since the distribution of allelopathic agents in nature varies both horizontally and vertically (Patrick et al., 1964; Blum and Rice, 1969; Patrick, 1971; Dao, 1987), it is unlikely that whole root systems are subject to the impact of allelopathic agents at any given point in time, thus the importance of this observation. Furthermore, our results show limited or no compensation for exposure to FA by unexposed roots.

We initially suspected, however, that the fraction of cucumber-seedling roots that were not in contact with FA would compensate for those that were in contact with FA by increasing root growth and/or increasing uptake rates. Indirect evidence for such compensation has been provided by Buchholtz (1971). Buchholtz noted that when nutrient solution was supplied to two seminal roots of corn growing in quackgrass-infested soil, the corn growth greatly improved. This led Buchholtz to conclude that the allelopathic effects of quackgrass on nutrient uptake of corn were predominantly localized rather than systemic.

Compensation was noted in roots not exposed to FA for net water uptake as the fraction of the roots in contact with FA increased. The lower net water uptake by roots in contact with FA and the decreasing fraction of the roots not in contact with FA, however, overwhelmed the compensation observed. No compensation was noted for net P and K uptake by roots not in contact with FA for those in contact with FA. Uptake actually declined as the fraction of the roots in contact with FA increased. This may have related to carbon (i.e., energy) allocation. Blum and Rebbeck (1989) noted that exposure of the whole root system of cucumber seedlings to FA resulted not only in a reduction of photosynthesis, but also in a reduction in the proportion of carbon allocated to roots. It is not known, however, how carbon would be allocated among FA exposed and nonexposed roots. The fact that net P and K uptake increased for roots in contact with FA as the fraction of the roots in FA increased suggests that at least part of the carbon supply for these slower growing roots (Blum and Rebbeck, 1989) was being shifted to ion uptake.

These observations, if confirmed for other species and other phenolic acids, suggest that the impact of allelopathic phenolic acids on net uptake of P, K, and water is related to the fraction of the roots in contact with phenolic acids and that compensation for net P, K, and water uptake by roots not in contact with phenolic acids for those in contact with phenolic acids ranges from no compensation to very limited compensation.

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SURFACE HYDROCARBON COMPONENTS OF TWO SPECIES OF *Nasutitermes* FROM TRINIDAD[1]

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Abstract—Colonies of *Nasutitermes costalis* (Holmgren) and *N. ephratae* (Holmgren) were collected from five locations in Trinidad. Cuticular hydrocarbons were characterized by gas chromatography–electron impact mass spectrometry and quantified by capillary gas chromatography. Sixteen major components were identified; all but one component (12, 16-dimethyltriacontane) were common to both species. The methyl-branched hydrocarbons were predominant in *N. costalis*, while the majority of the hydrocarbon components in *N. ephratae* were *n*-alkanes. One hydrocarbon (11,15-dimethylheptacosane) was found in abundance in samples of *N. ephratae* from Trinidad but was not previously reported from collections of this species in Panama. In addition to the morphology of the soldiers and alates and the architecture of the arboreal nests, *N. costalis* and *N. ephratae* from Trinidad can easily be separated by chromatograms of the hydrocarbons. *N. costalis* has an enormous 13,17-dimethylhentriacontane peak (mean = 42.4% of total hydrocarbon). In *N. ephratae* this peak is much smaller and the 12,16-dimethyltriacontane peak is completely missing. *N. costalis* from Trinidad and *N. corniger* from Panama appear to have cuticular hydrocarbon profiles that are more similar to one another than are those of *N. ephratae* from Trinidad and Panama.

[1]Isoptera: Termitidae: Nasutitermitinae.

Key Words—Chemotaxonomy, Isoptera, Termitidae, Nasutitermitinae, cuticular hydrocarbons, Neotropical termites, *Nasutitermes costalis*, *Nasutitermes corniger*, *Nasutitermes ephratae*, Caribbean insects.

INTRODUCTION

The tropicopolitan genus *Nasutitermes* Dudley is the largest genus in the Isoptera, containing approximately 212 species worldwide (based on compiled lists in Snyder, 1949; Roonwal, 1962; Araujo, 1977). Taxonomic discrimination is based on morphological characters that are often subtle and variable or most prominent in imagoes, which are rarely collected. As part of a broad examination of the taxonomy of Neotropical *Nasutitermes* in progress by B.L. Thorne, this study was initiated with two specific goals. First, we planned to compare the hydrocarbon components of *N. ephratae* (Holmgren) collected in Trinidad with those of a Panamanian population near the opposite end of the recognized species distribution (Howard et al., 1988). Second, we wished to examine hydrocarbon patterns for *N. costalis* (Holmgren) collected near its Type locality (Trinidad) and contrast those with hydrocarbon profiles of *N. corniger* (Motschulsky) collected near its Type locality (Panama, as reported in Howard et al., 1988).

Soldiers of *N. corniger* and *N. costalis* are currently impossible to discriminate without locality information. Nest architecture is also indistinguishable. When collections are made outside of the confirmed *N. corniger range* (Central America; see Thorne, 1980) or away from the recognized *N. costalis* distribution (Caribbean islands: Snyder, 1949; Araujo, 1977), species determination is equivocal. There is currently confusion over the extent of the distribution of each of these species in northern South America, with overlapping reports of each, and identifications of each from as far as Bolivia and Brazil (Roonwal and Rathore, 1976; reviewed in Thorne, 1980). In both the present report and in the Howard et al. (1988) study, the termites were collected by B.L. Thorne (supplemented in the latter project by material collected by S.C. Levings). Field collection methods and observations of nest architecture are thus directly comparable between the two locations.

In the past we have been successful using cuticular hydrocarbons to sort the morphologically variable species of dampwood termites, *Zootermopsis* spp., into distinct, consistent hydrocarbon phenotypes (Haverty et al., 1988). These chemical phenotypes were then used to identify morphological characters for unequivocal separation of nonsoldier morphs (castes) (Thorne and Haverty, 1989). Furthermore, we used these hydrocarbon phenotypes to identify two subspecies of *Z. nevadensis* (Hagen), *Z. n. nevadensis* (Hagen) and *Z. n. nuttingi* (Haverty and Thorne), on the basis of agonistic behavior (Haverty and Thorne, 1989). In broad terms, we sought here to supplement the data base on

surface hydrocarbons in *Nasutitermes* (Howard et al., 1988) and to see if this information (Howard and Blomquist, 1982) would help us resolve diagnostic problems within the genus. We report here the results of the characterization and quantification of the surface hydrocarbons of *N. costalis* and *N. ephratae* from Trinidad.

METHODS AND MATERIALS

Termites were collected from five separate locations in Trinidad. Trinidad is a continental island in the southeast corner of the Caribbean, where the island arcs approach the South American mainland. Fourteen colonies of *N. costalis* were collected from the following sites: six colonies from William Beebe Biological Station at Simla, Arima Valley (10°42′N, 61°17′W), one from Caura Valley (10°44′N, 61°22′W), four from Mt. St. Benedict (10°40′N, 61°24′W), and three from Matura Beach (10°40′N, 61°02′W). Nine colonies of *N. ephratae* were collected from four locations: three from Caura Valley, two from Mt. St. Benedict, three from Matura Beach, and one from Aripo Savannah (10°37′N, 61°12′W). Entire arboreal nests, or large sections of nests, were collected in the field and confined in plastic bags. Within 24 hr, live insects were removed from the carton matrix by briskly tapping a portion of nest material over sorting trays. One hundered large (female) workers were selected with forceps for extraction of hydrocarbons. Additional workers and soldiers from each colony, preserved in alcohol, have been deposited as vouchers in the collection of the Museum of Comparative Zoology, Harvard University, Cambridge, Massachusetts. Species were determined on the basis of nest architecture and the pattern of setae on the tergal sclerites (Emerson, 1925; Thorne, 1980; Thorne and Levings, 1989).

Cuticular lipids were extracted in the field by immersing 100 large workers in 10 ml hexane for 10 min. Extracted workers also were preserved in alcohol as vouchers. Hexane extracts were returned to Massachusetts, then allowed to evaporate to dryness. Dry extracts were shipped to Berkeley, California, for chromatographic analysis. Extracts were redissolved in 10 ml hexane and the hydrocarbons separated from other components by pipetting the 10-ml extract and an additional 8 ml of hexane through 3 cm of activated BioSil-A in Pasteur pipet mini-columns. Hydrocarbon extracts were evaporated to dryness under a stream of nitrogen and redissolved in 30 μl of hexane for analysis.

Hydrocarbons from each sampled colony were quantified with a Hewlett Packard 5890 gas chromatograph, equipped with a fused silica capillary column (30 m × 0.2 mm ID, HP-1), operated in the split mode (with a split ratio of 8:1) with a flame ionization detector (FID). Components were separated by a temperature program from 150°C to 320°C at 3°/min with a final hold of 10 mins. Peaks were characterized by retention time, and areas under the curves

(=quantity of a particular component) were automatically quantified by a Hewlett Packard 3390A electronic integrator. The proportion of each hydrocarbon component was computed by summing the areas of the peaks of each hydrocarbon component to calculate the area of all hydrocarbon peaks and then dividing the peak area of each hydrocarbon by the total. Proportions were converted to percentages; then summary statistics were computed for each hydrocarbon for each species.

After hydrocarbon components were quantified by FID, all samples for each species were combined to ensure an adequate sample for mass spectral characterization of each hydrocarbon component. These termites contain low absolute quantities of cuticular hydrocarbons (Howard et al., 1988). One hundred workers is the minimum number for quantification of hydrocarbon peaks but is not a sufficient sample for mass spectral identification of minor hydrocarbon components. By combining samples for each species, we were able to obtain sufficient material to identify the hydrocarbon peaks and ignore those that were not hydrocarbons (mass spectra were not characteristics of aliphatic hydrocarbons). Hydrocarbons were identified by gas chromatography–electron impact mass spectrometry, using the same Hewlett Packard 5890 gas chromatograph equipped with a Hewlett Packard 5970B Mass Selective Detector interfaced with Hewlett Packard Chemstation computer. Operating conditions were the same as those used for FID analyses.

Electron impact (EI) mass spectra were obtained at 70 eV. Retention times and mass spectra of extracted *n*-alkanes were compared with an external standard mixture (n-C_{22}, n-C_{24}, n-C_{28}, and n-C_{32}). Methyl-branched alkanes were tentatively identified by calculating their equivalent chain lengths (ECL) (Jackson and Blomquist, 1976). Mass spectra of methylalkanes were interpreted as described by Blomquist et al. (1987).

RESULTS AND DISCUSSION

The extraction technique that has worked so effectively with other species of termites (Howard et al., 1978, 1980a, b, 1982; Haverty et al., 1988, 1990) was not totally satisfactory with these *Nasutitermes*. Usually the hexane extract that has passed through the mini-column contains only hydrocarbons. In our samples numerous other components, presumably terpenoid defense secretions, coeluted with the hydrocarbons and may have masked some minor hydrocarbon components. However, comparison of our results with those of Howard et al. (1988) for *N. ephratae* and *N. corniger* indicates that only very minor hydrocarbon components might have been missed.

GC-MS analyses of the cuticular hydrocarbons of *N. costalis* and *N. ephratae* from Trinidad show that each species has hydrocarbon mixtures that are

qualitatively and quantitatively distinct from one another. Sixteen major hydrocarbon components were characterized (Table 1). Hydrocarbon components consist of a homologous series of *n*-alkanes (from *n*-C_{21} through *n*-C_{30}), two isomeric series of internally branched monomethyl alkanes (11-; 13-; 15-methylnonacosane and 11-; 13-; 15-methylhentriacontane), and a series of internally branched dimethylalkanes (11,15-dimethylnonacosane, 13,17-dimethylnonacosane, 11,15-dimethylhentriacontane, and 12,16-dimethyltriacontane). We identified no unsaturated components. One component was found only in *N. costalis*: 12,16-dimethyltriacontane (12,16-DimeC_{30}) (Table 2).

The majority of the hydrocarbons of *N. ephratae* (mean of 56.7%) are *n*-alkanes, whereas the *n*-alkanes comprise less than 30% of the hydrocarbon mixture of *N. costalis* (Tables 2 and 3). Within the *n*-alkane portion of the hydrocarbon profiles of these species, both appear to partition the various components similarly; C_{25} is the predominant *n*-alkane (Table 3). The apportionment of the branched alkanes is dramatically different between the two species. In *N. ephratae* the predominant branched hydrocarbons have a parent chain with 29 carbons, whereas in *N. costalis* the predominant branched alkane has a par-

TABLE 1. HYDROCARBONS IDENTIFIED FROM LARGE FEMALE WORKERS OF *Nasutitermes costalis* AND *N. ephratae* FROM TRINIDAD

Hydrocarbon	ECL[a]	CN[a]	Diagnostic MS ions
n-Heneicosane	21.00	21	296
n-Docosane	22.00	22	310
n-Tricosane	23.00	23	324
n-Tetracosane	24.00	24	338
n-Pentacosane	25.00	25	352
n-Hexacosane	26.00	26	366
n-Heptacosane	27.00	27	380
11,15-Dimethylheptacosane	27.63	29	168/169, 196/197, 239, 267
n-Octacosane	28.00	28	394
n-Nonacosane	29.00	29	408
11-; 13-; 15-Methylnonacosane[b]	29.32	30	168/169, 280/281; 196/197, 252/253; 224/225
11,15-; 13,17-Dimethylnonacosane[b]	29.61	31	168/169, 224/225, 239, 295; 196/197, 267
n-Triacontane	30.00	30	422
12,16-Dimethyltriacontane	30.60	32	182/183, 224/225, 253, 295
11-; 13-; 15-Methylhentriacontane[b]	31.33	32	168/169, 308/309; 196/197, 280/281; 224/225, 252/253
13,17-Dimethylhentriacontane	31.64	33	196/197, 224/225, 267, 295

[a] ECL = equivalent chain length, CN = carbon number.
[b] An isomeric mixture or two or more components coelute in this peak.

TABLE 2. MEAN PERCENT HYDROCARBON AND STANDARD DEVIATION FROM LARGE FEMALE WORKERS OF *Nasutitermes costalis* AND *N. ephratae* FROM TRINIDAD

	N. costalis (14 colonies)					*N. ephratae* (9 colonies)				
Hydrocarbon[a]	$\bar{X}$	SD	Min. value[b]	Max. value	Proportion ≤0.5% of total[c]	$\bar{X}$	SD	Min. value[b]	Max. value	Proportion ≤0.5% of total[c]
n-C_{21}	2.07	1.74	0.00	5.22	0.29	5.51	3.64	0.00	12.64	0.11
n-C_{22}	2.34	2.18	0.00	5.46	0.36	6.97	4.43	0.00	13.84	0.11
n-C_{23}	1.79	1.66	0.00	4.17	0.36	5.91	4.36	0.00	14.63	0.11
n-C_{24}	2.84	1.79	0.00	5.77	0.07	7.92	4.26	3.71	16.47	0.00
n-C_{25}	6.03	3.02	2.84	14.19	0.00	11.58	7.08	5.33	27.31	0.00
n-C_{26}	2.15	1.66	0.00	5.72	0.07	4.12	4.09	0.00	13.98	0.11
n-C_{27}	2.57	2.13	0.00	6.93	0.29	5.26	4.31	0.00	10.64	0.33
11,15-DimeC_{27}	1.95	1.89	0.00	5.32	0.29	6.45	4.90	0.00	15.57	0.22
n-C_{28}	1.05	1.42	0.00	3.48	0.57	1.41	2.21	0.00	5.04	0.67
n-C_{29}	3.94	2.89	0.00	8.32	0.14	2.30	2.49	0.00	6.28	0.44
11-; 13-; 15MeC_{29}	4.17	1.19	2.11	6.51	0.00	13.82	11.16	0.00	33.52	0.22
11,15-; 13,17-DimeC_{29}	8.39	3.41	0.00	13.59	0.07	16.06	5.17	7.83	24.69	0.00
n-C_{30}	4.67	2.27	0.00	7.96	0.07	5.69	5.77	0.00	14.27	0.44
12,16-DimeC_{30}	9.09	4.98	0.00	17.25	0.07	0.00	0.00	0.00	0.00	1.00
11-; 13-; 15-MeC_{31}	4.56	2.86	0.00	8.54	0.21	2.57	4.54	0.00	13.24	0.67
13,17-DimeC_{31}	42.42	10.98	20.35	59.30	0.00	4.43	7.31	0.00	18.62	0.67

[a] Hydrocarbons are the same as those reported in Table 1. Carbon number (C_n) is the total number of carbons in the parent chain, excluding the methyl groups.

[b] Values not necessarily zero but may have very low quantity (≤0.5% of the total hydrocarbon) or were simply not detectable for a given GLC analysis.

[c] Proportion of the chromatograms where a given hydrocarbon was not detectable.

TABLE 3. MEAN PERCENTAGE AND STANDARD DEVIATION OF EACH HYDROCARBON WITHIN SAME HYDROCARBON CLASS AND MEAN PERCENTAGE AND STANDARD DEVIATION OF *n*-ALKANES AND METHYL-BRANCHED ALKANES FROM LARGE FEMALE WORKERS OF *N. costalis* AND *N. ephratae* FROM TRINIDAD

	N. costalis (14 colonies)		*N. ephratae* (9 colonies)	
Hydrocarbon[a]	$\bar{X}$	SD	$\bar{X}$	SD
n-Alkanes				
n-C_{21}	6.03	4.44	8.95	4.36
n-C_{22}	6.36	5.12	11.52	5.82
n-C_{23}	4.80	3.99	9.33	4.98
n-C_{24}	9.02	3.82	14.15	6.32
n-C_{25}	26.23	22.21	19.50	6.56
n-C_{26}	6.54	3.21	7.06	5.17
n-C_{27}	7.09	4.72	10.03	7.82
n-C_{28}	2.43	3.18	2.42	3.70
n-C_{29}	12.50	9.50	4.00	4.12
n-C_{30}	19.00	15.21	13.03	15.59
Branched alkanes				
11,15-DimeC_{27}	2.86	2.86	12.90	8.48
11-; 13-; 15-MeC_{29}	6.08	1.70	27.34	18.65
11,15-; 13,17-DimeC_{29}	11.64	3.93	46.20	31.50
12,16-DimeC_{30}	12.49	6.12	0.00	0.00
11-; 13-; 15-MeC_{31}	6.97	4.09	5.03	8.60
13,17-DimeC_{31}	59.96	5.22	8.53	13.64
Total *n*-alkanes	29.44	16.52	56.67	18.07
Total branched alkanes	70.56	16.52	43.33	18.07

[a]Hydrocarbons are the same as those reported in Table 2.

ent chain of 31 carbons (Table 3). Probably the most obvious difference, for chemotaxonomic discrimination of these two species, is the disparity between the comparatively large amount of 13,17-DimeC_{31} in *N. costalis* and the apparent absence of 12,16-DimeC_{30} in *N. ephratae*. This pattern was consistent in all 23 sampled colonies.

One of the species we examined from Trinidad, *N. ephratae*, was also examined by Howard et al. (1988) from Panama. These two localities are near the eastern and western extremes of its distribution. Striking similarities in morphology of both soldiers and primary reproductives and in arboreal nest architecture make us confident that specimens from Panama and Trinidad belong to the same "morphospecies" (Thorne, 1980). Specimens from these locations have been determined as such by both T.E. Snyder and A.E. Emerson, who examined material from both localities, although they did not collect first-hand.

Based on preliminary data, genera of staphylinid termitophiles (*Perinthus* and *Termatogaster*) found in *N. ephratae* nests from Panama and Trinidad overlap in part, but the beetle species are different (Thorne, in preparation).

Howard et al. (1988) reported numerous terminally branched monomethyl components that occurred in very low abundance ($\leq 1.85\%$ of the methyl-branched components) in Panamanian *N. ephratae*. These components may have been present in our samples but in concentrations below the detectability of our GLC system, or they may have been masked by the terpenoid defense secretions. Nonetheless, the very low abundance of these components renders them inadequate as taxonomic characters (Haverty et al., 1989; Page et al., 1990).

We observed additional significant qualitative differences between the Trinidad and Panama populations of *N. ephratae*. Howard et al. (1988) reported considerable quantities of 12,16-DimeC_{30} in *N. ephratae*; we never identified this component in this species from Trinidad. In addition, we identified a plentiful component (11,15-DimeC_{27}) in both *N. ephratae* and *N. costalis* from Trinidad that was not identified in either *N. ephratae* or *N. corniger* from Panama. The homolog of this component (11,15-DimeC_{29}) was identified in significant amounts from the three *Nasutitermes* from Panama and Trinidad and is the predominant component of the cuticular hydrocarbon profile of *N. ephratae* from Trinidad.

Hydrocarbon profiles of *N. corniger* from Panama and *N. costalis* from Trinidad are more similar than are the hydrocarbon patterns of *N. ephratae* from Panama and Trinidad. Not only do *N. costalis* and *N. corniger* share the same hydrocarbon components, with the exception of the minimally abundant 11,15-DimeC_{27} ($\leq 2.9\%$ of the branched-hydrocarbon component), but they apparently possess the two predominant dimethyl alkanes, 12,16-DimeC_{30} and 13,17-DimeC_{31}, in roughly equivalent proportions: 12.5 vs 14.2 and 60.0 vs 55.9% of the methyl-branched alkanes, respectively (Table 3) (Table 2; Howard et al., 1988). Based on these analyses, it appears that *N. costalis* from Trinidad and *N. corniger* from Panama, two "distinct" species, are more similar in terms of surface hydrocarbons than are populations of *N. ephratae* from these same locations. Morphology and nest architecture of *N. costalis* and *N. corniger* are also quite similar and are currently being examined in detail (Thorne, in preparation).

This present study represents only the third report of the cuticular hydrocarbons of species of *Nasutitermes*, one of the most ubiquitous and abundant pantropical groups of termites. Moore (1969) provided the first preliminary description of the cuticular lipids of the Australian termite, *N. exitiosus* (Hill). He reported that the unsaturated component comprised the majority of the cuticular lipids. Analysis of the saturated fraction revealed normal paraffins from C_{24} to C_{47}. No methyl-branched components were described. His results are drastically different from those reported here or by Howard et al. (1988).

Similarities among the hydrocarbon mixtures of the three species of *Nasutitermes* most recently examined, here and by Howard et al., (1988), *and* the apparent qualitative and quantitative differences in hydrocarbon profiles of *N. ephratae* from the extremes of the species' distribution raise some interesting questions. Are cuticular hydrocarbons valuable chemotaxonomic characters for all *Nasutitermes* species or do hydrocarbon profiles show intraspecific variability, as has been found with defense secretion components (Gush et al., 1985)? In this study, the hydrocarbon component (11,15-DimeC_{27}) is the homolog to a component (11,15-DimeC_{29}) found in all of the investigated *Nasutitermes* species from both localities. Because of this close chemical similarity, the presence or absence of the additional variant (11,15-DimeC_{27}) might be due to a minor polymorphism between the two *N. ephratae* populations.

As has been found with other taxonomic character systems, hydrocarbon profiles may not show exact qualitative and/or quantitative species specificity, especially across a broad geographic range. Further sampling within *Nasutitermes* and in other groups should be encouraged to determine the prevalence of such variability and to ascertain its biological and taxonomic relevance.

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DEFENSE OF PARSNIP WEBWORM AGAINST PHOTOTOXIC FURANOCOUMARINS: ROLE OF ANTIOXIDANT ENZYMES

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Abstract—The parsnip webworm, *Depressaria pastinacella* (Lepidoptera: Oecophoridae), feeds on plants rich in furanocoumarins, phototoxic allomones. Final-instar larvae possess high levels of activities of antioxidant enzymes (superoxide dismutase, catalase, glutathione reductase), which detoxify oxygen radicals generated from the furanocoumarins of their host plants. When added to an artificial diet, three linear furanocoumarins (xanthotoxin, bergapten, imperatorin) do not increase levels of the antioxidant enzymes. However, on diets containing both xanthotoxin and piperonyl butoxide, a cytochrome P-450 inhibitor, food utilization indices of the insect are reduced and superoxide dismutase activity is enhanced. These data suggest that cytochrome P-450s act as a primary detoxification system of ingested furanocoumarin, and antioxidant enzymes as a backup system to detoxify oxygen radicals generated by unmetabolized furanocoumarins.

Key Words—Parsnip webworm, *Depressaria pastinacella*, Lepidoptera, Oecophoridae, antioxidant enzymes, superoxide dismutase, catalase, glutathione reductase, furanocoumarins, synergist, detoxification, defense.

INTRODUCTION

Furanocoumarins from the plant families Apiaceae (Umbelliferae) and Rutaceae possess toxicity toward various organisms ranging from bacteria to mammals (Murray et al., 1982). The mechanism of furanocoumarin toxicity is believed to be photoactivation by near-UV light (UVA, 320–400 nm). Photoactivated

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furanocoumarins directly conjugated with pyrimidine bases of DNA to form irreversible cycloadducts that interfere with DNA transcription and translation (Song and Tapley, 1979). Photoactivated furanocoumarins also bind to unsaturated fatty acids (Specht et al., 1988) and proteins (Yoshikawa et al., 1979). In addition, photoactivated furanocoumarins can interact with molecular oxygen to form reactive oxygen species such as singlet oxygen and superoxide radicals (Joshi and Pathak, 1983), which cause oxidative stress through lipid peroxidation and enzyme inactivation (Bus and Gibson, 1979).

The parsnip webworm, *Depressaria pastinacella* (Lepidoptera: Oecophoridae), feeds on members of only three genera of the Apiaceae (Hodges, 1974), all of which are rich in furanocoumarins (Murray et al., 1982). Growth and development of the parsnip webworm are not affected by ingestion of xanthotoxin, a linear furanocoumarin (Nitao, 1989; Berenbaum et al., 1989). Nitao (1989) also demonstrated that cytochrome P-450 monooxygenases (P-450s) are involved in xanthotoxin metabolism and that the P-450s that metabolize xanthotoxin are inducible by xanthotoxin itself in the parsnip webworm.

Despite the rapid detoxification of furanocoumarins by P-450s in the parsnip webworm, all furanocoumarins ingested are not broken down (Nitao, 1987). Some furanocoumarins that escape metabolic detoxification might produce toxic oxyradicals, a potential threat to living cells (Lee and Berenbaum, 1989). The threat of toxic radicals can be eliminated by the action of a suite of antioxidant enzymes (Larson, 1986). The antioxidant enzymes—superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPOX), and glutathione reductase (GR)—remove radicals produced not only by endogenous activities such as electron transport and oxidoreductase reaction (Fridovich, 1983), but also by exogenous sources such as ingested plant phototoxins (Lee and Berenbaum, 1989). Thus, these antioxidant enzymes are considered to be particularly important in species adapted to feed on phototoxin-containing plants (Pardini et al., 1989; Lee, 1989).

The purpose of this study was to evaluate the adaptive role of antioxidant enzymes in host utilization by a specialist insect. We studied antioxidant enzyme activities of *D. pastinacella*, and their inducibility by UVA radiation and ingestion of furanocoumarins. We also examined the effects of piperonyl butoxide, a synthetic cytochrome P-450 inhibitor (Metcalf, 1967), on utilization of food containing furanocoumarin, and on the corresponding activities of antioxidant enzymes in order to investigate the possible interactions between the cytochrome P-450s and antioxidant enzymes.

METHODS AND MATERIALS

Chemicals. Xanthotoxin (8-methoxypsoralen) and bergapten (5-methoxypsoralen) were purchased from Sigma Chemical Co., St. Louis, Missouri, and from Aldrich Chemical Co., Milwaukee, Wisconsin. Imperatorin (8-isoamy-

leneoxypsoralen) was purchased from Serva Feinbiochemica, Heidelberg, West Germany. Reagents for enzyme assays, unless otherwise noted, were purchased from Sigma.

Insects. Parsnip webworm pupae were collected in July 1987 from wild parsnip (*Pastinaca sativa*) stalks at two sites in Champaign County, Illinois. A laboratory colony was maintained on artificial medium as described by Nitao and Berenbaum (1988).

Effects of Furanocoumarins on Antioxidant Enzyme Activities. Xanthotoxin, bergapten, and imperatorin (Figure 1) were individually dissolved in ether and directly incorporated into artificial diets. Small cubes of furanocoumarin-containing diets were prepared as described in Lee and Berenbaum (1989). The final concentrations of furanocoumarins were 0.15% and 0.6% wet weight, which, respectively, correspond to average and maximum levels measured in natural host-plant populations (Berenbaum et al., 1984, 1986; Nitao and Zangerl, 1987). The same volume of solvent was incorporated into control diets. Two newly molted sixth-instar larvae were placed in a plastic cup (about 28 cc) with a diet cube, and the cup was capped with a clear plastic Petri-dish cover, which allows transmission of UVA and visible radiation. In order to take into account photoactivation of furanocoumarins, which can greatly enhance toxicity to insects (Berenbaum, 1978), for each chemical 16 larvae were exposed to a sunlight-simulated spectrum (black light and cool-white bulbs, 16-hr photoperiod) and another 16 larvae were protected from UV light with an opaque Plexiglas filter (3 mm in thickness) which does not transmit wavelengths below 400 nm. UVA intensity was 250 $\mu W/cm^2$, which is much lower than that of a summer day but close to the actual range of UVA intensity in the silk-webbed feeding habitat of the parsnip webworm in nature (K. Lee, personal observation). For each chemical and concentration, larvae were fed ad libitum for 48 hr on diets at 27°C. The larvae were collected, frozen with liquid N_2, and stored in a freezer (−70°C) for enzyme assays.

Effects of Piperonyl Butoxide and Furanocoumarin on Food Utilization and Antioxidant Enzyme Activities. Piperonyl butoxide (PB: ICN Pharmaceuticals, Plainview, New York) and xanthotoxin (xan) were dissolved individually in acetone and directly incorporated into liquid diets together or alone at concentrations of 0.01% and 0.15%, respectively. Diet cubes containing these

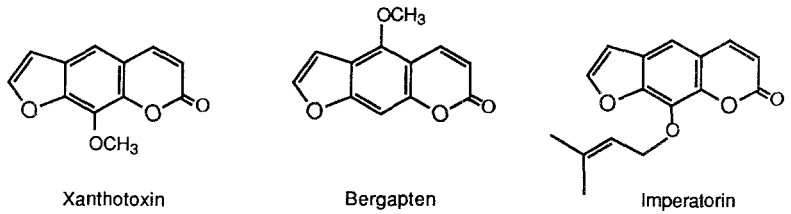


FIG. 1. Structures of furanocoumarins tested.

chemicals were prepared as described for the earlier experiment. Each newly molted final-instar larva was placed individually and fed for 48 hr on a diet cube in a Petri dish (5 cm in diameter) under UVA radiation. Twenty larvae were taken from each of the four treatments (xan, xan + PB, PB, control) for antioxidant enzyme assays. Remaining larvae (13–15 individuals per treatment) were separated from diet and starved for 3 hr to empty their guts. Larvae, remaining diet cubes, and feces were oven-dried (60°C) for six days and weighed. In order to obtain a wet weight–dry weight conversion ratio at the onset of each trial, 10 newly molted final-instar larvae from the same cohort as experimental larvae and 10 diet cubes from each diet batch were dried and weighed. Weight gain (WG) and food utilization indices (RCR, RGR, ECI, ECD) were calculated on a dry-weight basis (Waldbauer, 1968). Thus, RCR = relative consumption rate, RGR = relative growth rate, ECI = efficiency of conversion of ingested food to biomass, and ECD = efficiency of conversion of digested food to biomass.

Assay of Antioxidant Enzyme Activity. For each treatment, four larvae were homogenized in 4 ml of 50 mM potassium phosphate buffer (pH 7.8) in an ice-chilled glass tissue grinder. Homogenates were centrifuged at 850*g* for 15 min or 3000*g* for 30 min. The supernatant was used immediately thereafter for antioxidant enzyme assays. In order to prevent the enzyme source from oxidizing (surface darkening) during the assay, the ice bucket in which enzyme sources were placed was saturated with N_2. Each enzyme assay was conducted in duplicate, and the average value was used in calculating mean values. Each experimental treatment was replicated at least three times.

Activities of three antioxidant enzymes (SOD, CAT, GR) were measured as described in Lee and Berenbaum (1989), and protein content was determined by the method of Lowry et al. (1951).

Statistical Analysis. Differences in enzyme activities among treatments in the first experiment were evaluated by three-way ANOVA, with main effects of chemical, dose, and light. Differences in food utilization indices and enzyme activities among treatments in the second experiment were assessed by one-way ANOVA. When the ANOVA F statistic was significant ($P < 0.05$), Tukey's multiple range test was used to compare treatment means. Values for ECI (%) and ECD (%) were transformed (arcsine square root) to equalize variance prior to statistical analysis.

RESULTS

Effects of Furanocoumarins on Antioxidant Enzyme Activities. The furanocoumarins xanthotoxin, bergapten, and imperatorin showed neither inductive nor inhibitory effects on antioxidant enzyme activities (Table 1). Neither

TABLE 1. EFFECTS OF FURANOCOUMARINS AND UVA RADIATION ON ANTIOXIDANT ENZYME ACTIVITIES[a] OF FINAL-INSTAR LARVAE OF *Depressaria pastinacella*[b]

Treatment	UV	SOD	CAT	GR
Control	+	2.82 ± 0.29	573.4 ± 35.8	2.49 ± 0.17
	−	2.65 ± 0.30	585.2 ± 41.1	2.73 ± 0.25
Xanthotoxin 0.15%	+	2.98 ± 0.27	637.8 ± 27.8	2.49 ± 0.11
	−	3.01 ± 0.27	565.4 ± 24.2	2.52 ± 0.21
Xanthotoxin 0.6%	+	2.96 ± 0.30	605.9 ± 27.1	3.00 ± 0.20
	−	2.85 ± 0.22	593.0 ± 36.9	2.83 ± 0.21
Bergapten 0.15%	+	2.65 ± 0.23	555.3 ± 29.9	2.78 ± 0.09
	−	2.75 ± 0.15	558.6 ± 26.4	2.53 ± 0.08
Bergapten 0.6%	+	2.79 ± 0.31	559.6 ± 26.3	2.58 ± 0.20
	−	2.86 ± 0.22	602.5 ± 21.3	2.49 ± 0.23
Imperatorin 0.15%	+	2.79 ± 0.19	539.6 ± 28.1	2.49 ± 0.17
	−	2.78 ± 0.18	545.0 ± 27.0	2.71 ± 0.25
Imperatorin 0.6%	+	2.82 ± 0.19	574.8 ± 27.0	2.46 ± 0.26
	−	2.93 ± 0.11	571.0 ± 32.3	2.68 ± 0.22

[a] The 850*g* supernatant of whole body homogenate was used for enzyme source. Units of activities: SOD (units/mg protein/min), CAT (μmol H_2O_2 decomposed/mg protein/min), GR (nmol NADPH oxidation/mg protein/min). Enzyme activity means ± SE were obtained from four experiments, each with duplicates.

[b] Threeway ANOVA showed no significant main effects on interactions at $P = 0.05$.

increasing furanocoumarin concentration nor exposure to UVA affected the activities.

Effects of Piperonyl Butoxide and Furanocoumarin on Food Utilization and Antioxidant Enzymes. Dietary xanthotoxin had no negative effect on the food utilization of *D. pastinacella* larvae (Table 2). Piperonyl butoxide (PB) alone also did not affect the food utilization. However, when the larvae were fed on diets containing both xanthotoxin (0.15%) and PB (0.01% wet weight), WG, RGR, ECI, ECD were significantly ($P < 0.05$) reduced, but RCR was increased (Table 2).

Antioxidant enzyme activities were not affected by either dietary xanthotoxin or PB. Dietary xanthotoxin with PB, however, significantly increased (1.5-fold) the SOD activity ($P = 0.01$) (Table 3).

DISCUSSION

Role of Antioxidant Enzymes in Resistance to Furanocoumarins. Despite the generally rapid detoxification of furanocoumarins by P-450s in *D. pastinacella* (Nitao, 1989), some portion of ingested furanocoumarin may bypass the

TABLE 2. FOOD UTILIZATION OF *Depressaria pastinacella* REARED ON DIETS CONTAINING XANTHOTOXIN (XAN, 0.15% WET WEIGHT) AND PIPERONYL BUTOXIDE (PB, 0.01% WET WEIGHT) UNDER UVA RADIATION

	Treatments[a]			
Indices	Control	PB	Xan	Xan + PB
WG	22.0 ± 0.9a	19.5 ± 0.5a	19.2 ± 0.9a	14.8 ± 1.1b
RCR	2.10 ± 0.06b	2.27 ± 0.04ab	2.10 ± 0.13ab	2.38 ± 0.08a
RGR	0.44 ± 0.01a	0.43 ± 0.01a	0.42 ± 0.01ab	0.39 ± 0.01b
ECI (%)	21.2 ± 0.6a	19.1 ± 0.3ab	20.7 ± 1.3a	16.7 ± 0.9b
ECD (%)	48.1 ± 1.5a	44.0 ± 1.1ab	42.8 ± 3.4ab	39.8 ± 2.3b

[a]Each value indicates mean ± SE. Sample sizes of control, PB, xan, and xan + PB are 13, 11, 10, and 13, respectively. Values sharing the same letter are not significantly different at $P = 0.05$ according to Tukey's multiple range test. WG = weight gain, RCR = relative consumption rate, RGR = relative growth rate, ECI = efficiency of conversion of ingested food to biomass, ECD = efficiency of conversion of digested food to biomass, PB = piperonyl butoxide, Xan = xanthotoxin.

TABLE 3. ANTIOXIDANT ENZYME ACTIVITIES OF FINAL-INSTAR LARVAE OF *Depressaria pastinacella* FED ON DIETS CONTAINING XANTHOTOXIN (0.15% WET WEIGHT) AND PIPERONYL BUTOXIDE (0.01% WET WEIGHT) UNDER UVA

		Activities[a]		
Treatment	*N*	SOD	CAT	GR
Control	3	3.87 ± 0.16	780 ± 47.0	2.29 ± 0.07
PB	3	4.03 ± 0.13	760 ± 34.6	2.17 ± 0.15
Xanthotoxin	3	4.60 ± 0.08	688 ± 43.8	1.83 ± 0.29
Xan + PB	3	6.01 ± 0.22[b]	671 ± 38.3	1.71 ± 0.05

[a]The 3000*g* supernatant of whole body homogenate was used for enzyme source. Units of activities: SOD (unit/mg protein/min), CAT (μmol H_2O_2 decomposed/mg protein/min), GR (nmol NADPH oxidation/mg protein/min).

[b]Means significantly different from the control in the same column at $P = 0.01$ according to Tukey's multiple range test.

primary detoxification system. In experiments with labeled xanthotoxin, 5% of ingested xanthotoxin was found to be unmetabolized three days after ingestion in *D. pastinacella*, and unmetabolized xanthotoxin, along with its metabolites, was found in the silk and silk glands (Nitao, 1987). The small portion of unmetabolized furanocoumarins could produce toxic oxyradicals, which could be eliminated by the secondary defense system of antioxidant enzymes. The con-

stitutive level of SOD activity (2.82 units/mg protein/min) of *D. pastinacella* was close to that of *P. polyxenes*, another species adapted to feeding on furanocoumarin-containing plants (Lee, 1989), but threefold higher than that of *T. ni*, a generalist insect (Ahmad et al., 1987; Lee and Berenbaum, 1989). CAT and GR activities in *D. pastinacella* were two- to threefold, and fourfold higher than those of *P. polyxenes* and *T. ni*, respectively (Lee, 1989; Lee and Berenbaum, 1989). The high constitutive levels of antioxidant enzyme activities are likely to contribute in part to the resistance of *D. pastinacella* to furanocoumarins encountered in host plants.

Induction of antioxidant enzyme activity may be dependent upon the in vivo generation of oxyradicals from ingested phototoxins (see Lee and Berenbaum, 1989), which is determined by the extent of metabolic detoxification of phototoxins, the efficiency of oxyradical generation from the unmetabolized phototoxins, and the ability of activating wavelengths of light to reach the target phototoxins through the cuticle. Xanthotoxin and bergapten produce superoxide radicals photodynamically as well as singlet oxygen (Joshi and Pathak, 1983). However, there is no information on oxyradical generation of imperatorin, a linear furanocoumarin, which shows weak phototoxicity and photomutagenicity in algal cells (Schimmer and Abel, 1986), and generates singlet oxygen (De Mol et al., 1984). Nonetheless, the fact that antioxidant enzyme activities in *D. pastinacella* are unchanged by dietary furanocoumarins suggests that high constitutive levels of SOD, CAT, and GR activities in *D. pastinacella* may be sufficient to detoxify toxic oxygen radicals generated by whatever fraction of furanocoumarins escape the high activity of detoxification enzymes such as P-450s.

Evidence for Consequential Defenses by P-450s and Antioxidant Enzymes. Since P-450s are responsible for rapid detoxification of furanocoumarin in *D. pastinacella* larvae (Nitao, 1989), in vivo treatment with a P-450 inhibitor may slow down the detoxification of furanocoumarin. In vivo effects of PB on xanthotoxin-metabolizing P-450s of *D. pastinacella* have not been investigated in this study, although in vitro inhibition of P-450 activities by PB has been found in *D. pastinacella* (Nitao, 1989). Nonetheless, the induction of SOD by xanthotoxin and PB treatment in *D. pastinacella* suggests that PB-inhibited P-450s allow ingested xanthotoxin to produce an excess of oxyradicals, which in turn induces SOD activity.

The case of *D. pastinacella* with suppresed P-450 activity by PB is quite similar to that of *T. ni*, a generalist noctuid that is less likely to encounter xanthotoxin in its host range. Xanthotoxin-related P-450 activity in *T. ni* is over 200 times lower than that of *D. pastinacella*, and SOD activity in *T. ni* is threefold lower than that of *D. pastinacella*, but inducible (about threefold) by dietary xanthotoxin (Lee and Berenbaum, 1989). Since P-450 cannot process xanthotoxin rapidly in both *T. ni* (Lee and Berenbaum, 1989) and PB-treated *D.*

pastinacella (this study), unmetabolized xanthotoxin that escapes the P-450 action may accumulate in the body and generate toxic oxyradicals which, in turn, induce the antioxidant enzyme. These comparative studies suggest a hypothesis that induction of antioxidant enzymes by phototoxins occurs when low P-450 activity toward the phototoxins permits the accumulation of oxidative stress from unmetabolized phototoxin (Figure 2). These interactive enzyme systems were accordingly called "consequential defenses" (Lee and Berenbaum, 1989). P-450s act as a primary detoxification system of ingested furanocoumarin, and antioxidant enzymes act as a backup system to detoxify oxyradicals produced by the fraction of unmetabolized furanocoumarin.

Synergistic effects of PB were apparent in the food utilization studies (Table 2). Significantly lowered food utilization indices in the xanthotoxin + PB treat-

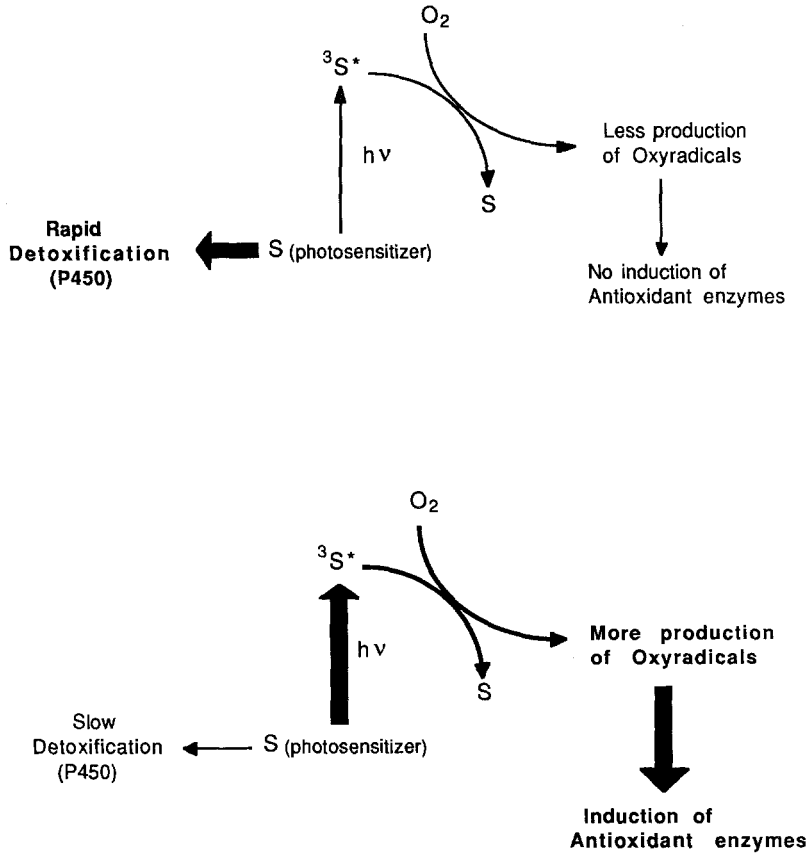


FIG. 2. A proposed hypothesis for antioxidant enzyme induction.

ment were apparently due to delayed xanthotoxin detoxification by PB-inhibited P-450s.

The induction of SOD activity by xanthotoxin + PB invites speculation on the ecological situation, since, in host plants of *D. pastinacella* such as wild parsnip, furanocoumarins cooccur with methylenedioxyphenyl-containing compounds such as myristicin and safrole, which inhibit P-450 activity (Berenbaum and Neal, 1985). Despite the presence of cooccurring phytosynergists (Neal, 1989) in the host plants, *D. pastinacella* can feed on furanocoumarin-containing plants due in part to an inducible antioxidant enzyme that eliminates excessive oxyradicals generated by the interaction of phytosynergists cooccurring with phototoxic furanocoumarins and primary detoxification systems.

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SEX PHEROMONE OF A *Planotortrix excessana*[1] SIBLING SPECIES AND REINVESTIGATION OF RELATED SPECIES

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Abstract—The sex pheromone of a *Planotortrix excessana* sibling species was investigated. Females were found to produce eight potential pheromone components: dodecyl acetate, tetradecyl acetate (14:OAc). (*Z*)-5-tetradecenyl acetate (Z5-14:OAc), (*Z*)-7-tetradecenyl acetate (Z7-14:OAc), (*Z*)-9-tetradecenyl acetate, hexadecyl acetate, (*Z*)-7-hexadecenyl acetate, and (*Z*)-9-hexadecenyl acetate. When these compounds were bioassayed using field-trapping and wind-tunnel techniques, only 14:OAc, Z5-14:OAc, and Z7-14:OAc were found to be behaviorally active. The sex pheromone glands of females of other species including, *Planotortrix* "MBS," *Planotortrix* "M," *P. notophaea*, *Ctenopseustis servana*, and a *C. obliquana* sibling species, were also found to contain Z5-14:OAc or Z7-14:OAc, singly or in combination. In the case of *Planotortrix* "M," the addition of Z7-14:OAc to the previously identified sex pheromone blend of Z5-14:OAc and 14:OAc was found to increase trap captures of male moths of this species. Thus in these New Zealand species (and in some Australian species), Z5-14:OAc and Z7-14:OAc appear to be utilized in combination in pheromonal communication just as (*Z*)-11-tetradecenyl acetate and (*E*)-11-tetradecenyl acetate are used by many species of Holarctic Tortricidae in the tribe Archipini.

Key Words—*Planotortrix excessana*, *Ctenopseustis*, Lepidoptera, Tortricidae, sex pheromones, (*Z*)-5-tetradecenyl acetate, (*Z*)-7-tetradecenyl acetate, tetradecyl acetate.

[1]Lepidoptera: Tortricidae: Tortricinae.

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INTRODUCTION

The endemic New Zealand tortricid genera *Planotortrix* (Dugdale) and *Ctenopseustis* (Meyrick) contain several species that are major pests of fruit crops, and hence the biology of these species has been the subject of considerable study (Wearing et al., 1990). While species in these genera exhibit some unusual morphological character states (Horak, 1984), they are highly distinctive relative to most other tortricine Tortricidae in their sex pheromone components, (*Z*)-5-tetradecenyl acetate (*Z*5–14:OAc), (*Z*)-7-tetradecenyl acetate (*Z*7–14:OAc), and (*Z*)-8-tetradecenyl acetate (*Z*8–14:OAc). These three chemicals are often found in the sex pheromones of these species, singly or in the combinations *Z*5–14:OAc and *Z*8–14:OAc, or *Z*5–14:OAc and *Z*7–14:OAc (Foster and Dugdale, 1988). These three components are distinct from the Δ11-unsaturated tetradecenyl acetates, alcohols, or aldehydes commonly found in other species in the tribe Archipini (Roelofs and Brown, 1982).

One such taxon apparently utilizing one of these combinations in its sex pheromone is a *Planotortrix excessana* (Walker) sibling species. Galbreath et al. (1985) identified the sex pheromone of a *P. excessana* population from Christchurch (South Island) as a mixture of *Z*8–14:OAc and tetradecyl acetate (14:OAc). During the course of identifying the sex pheromone of this population, Galbreath et al. (1985) collected a single gravid female *P. excessana* from Tokoroa (mid North Island) and found that progeny from this female apparently utilized a different sex pheromone from the Christchurch population. Primarily by comparing retention times on a Carbowax 20 M capillary column they tentatively identified *Z*5–14:OAc and *Z*7–14:OAc as the most abundant compounds in the sex pheromone gland of these females. By collecting larvae from the field and analyzing the sex pheromone glands of the resultant female moths, Foster et al. (1986) found this particular sibling species of *P. excessana* to be distributed throughout much of the North, South and Stewart Islands of New Zealand.

In order to produce a suitable sex pheromone blend for monitoring the *Z*5–14:OAc/*Z*7–14:OAc *P. excessana* sibling species in orchards, we investigated the sex pheromone of this species in greater detail, which we report here. We also report the results of further studies on the sex pheromones of other species in the genera *Planotortrix* and *Ctenopseustis* that use one or both of these chemicals in their sex pheromone.

METHODS AND MATERIALS

Insects. P. excessana were collected as larvae at Rukuhia (near Hamilton, northern North Island), Appleby Research Orchard (northern South Island), and Dunedin (southern South Island) off various hosts. A laboratory colony of the

Rukuhia insects was established and built up over three generations before use in this study. Larvae collected from the other two regions were reared to pupae on synthetic diet (Singh, 1974) and the resultant female moths analyzed. For the various chemical and behavioral analyses, the pupae were sexed and the sexes maintained in separate incubators.

Planotortrix sp. (*Planotortrix* "M") found on mangrove, *Avicennia resinifera* Forst. f, were collected at the Firth of Thames (northern North Island) as larvae. In contrast to the last time we studied this species (Foster et al., 1987), the larvae were found to be extremely uncommon, and despite two extensive collecting trips only a small number of larvae were found. From these collections only five larvae were reared through to adult females.

Planotortrix sp. (*Planotortrix* "MBS") found on *Brachyglotis reinoldii*, Endl, were collected as larvae at Lee Bay (Stewart Island); *C. servana* were collected as larvae off *Corynocarpus laevigatus* J.R. et G. Foster, at Manaia, Taranaki (southwestern North Island); *C. obliquana* (Walker) were from a colony in our laboratory, originating from Lincoln (near Christchurch, mid-coastal South Island). *P. notophaea* were collected off *Podocarpus totara* G. Benn ex D. Don, cv. Golden Totara, at Manurewa, Auckland (northern North Island). *P. flammea* were collected off *Hebe stricta* var. *egmontia* L.B. Moore at Mt Egmont (Taranaki). All the larvae from these species were reared as above.

Chemical Analyses. Female moths were used two to four days after eclosion. The sex pheromone glands of females were everted approximately 1 hr into the scotophase period and dissected under a binocular microscope. The dissected glands were extracted by allowing them to stand in 10–20 μl of pentane for ca. 22 hr at ambient temperature. Tridecanyl acetate (9 ng) was added to the extracts as an internal standard.

Extracts from all but one of the species were analyzed by capillary gas chromatography (GC) using two columns, a very polar 60-m × 0.25-mm-ID SP2340 (Supelco Inc., Bellefonte, Philadelphia) and a polar 30-m × 0.32-mm-ID bonded phase Carbowax 20 M (Quadrex Corporation, New Haven, Connecticut). Extracts from pheromone glands of the two *P. flammea* females were analyzed using only the SP2340 column. A Varian 3500 gas chromatograph equipped with a splitless injector and a flame ionization detector was used for the GC analyses. The column oven was temperature programmed from 60 to 140°C at 8°C/min following an initial delay of 1 min, and then to 200°C at 2.5°C/min. Nitrogen was used as the carrier gas at a linear flow velocity of 10 cm/sec. This combination of columns and conditions allowed unequivocal assignment of virtually all monounsaturated *n*-tetradecenyl and *n*-hexadecenyl acetates (Heath et al., 1980).

P. excessana pheromone gland extracts were also analyzed by GC–mass spectrometry (GC-MS) using a Hewlett Packard 5890-VG Instruments 70SE GC-MS system. Electron ionization mass spectra were recorded at 70 eV. The gas chromatograph was fitted with a 30-m × 0.25-mm-ID SP2340 capillary

column (Supelco Inc.), which was temperature programmed as above. Helium at a linear flow velocity of 25 cm/sec was the carrier gas.

Field Trapping. Field-trapping for *P. excessana* was conducted at Lower Moutere as preliminary attempts to trap this species at Rukuhia failed to catch more than a few males. The appropriate chemical blends were loaded onto rubber septa as described, and placed inside delta-shaped sticky traps. The traps were hung on native shrubs approximately 1.5–2 m from the ground and at least 10 m apart from each other in a randomized complete block design. The trial was conducted from October 10, 1988, to January, 5 1989, and consisted of three replicates of each treatment plus blank traps. Field trapping for *Planotortrix* "M" was conducted in the mangrove swamp in the Firth of Thames. Traps were placed on mangrove bushes approximately 1 m from the ground. Data from the field trials were transformed to their $\sqrt{(X + 0.5)}$ values, subjected to an ANOVA, and the treatment means compared using either Duncan's multiple-range test (for *P. excessana*) or a two-way ANOVA (for *P.* "mangrove"). Traps in both studies were checked, males were counted, and traps were re-randomized weekly.

Wind Tunnel. A wind tunnel based on the design of Miller and Roelofs (1978) was used to bioassay chemical blends. Three- to six-day-old naive males were conditioned at 20°C and 0.5 lux (white light) for 1 hr before they were released individually into the tunnel. The experimental design was a randomized complete block with all the treatments in a particular experiment tested in random order each day. Two behaviors, orientation (casting across the tunnel mouth) and landing at the source, were recorded and analyzed by Fisher's protected LSD (Milliken and Johnson, 1984, p. 31). Two experiments were carried out, each consisting of five and four replicates, respectively, with each replicate consisting of between 10 and 22 males flown to each treatment.

The chemical blends were made up as pentane solutions, and the concentrations of the individual components checked by GC as above. The appropriate amount of a blend was loaded onto a rubber septum (Thomas Scientific) and the solvent allowed to evaporate at ambient temperature. The septum containing a particular blend was presented in the wind tunnel on a 10-cm × 10-cm aluminum table. When not in use, the septa were stored in glass vials at −15°C.

RESULTS

P. excessana. GC analysis, using the SP2340 column, of several pheromone gland extracts (5–10 glands) of females originally collected as larvae in Dunedin showed a consistent pattern of peaks (amounts relative to the total amount of Z5–14 : OAc and Z7–14 : OAc expressed as 100) with retention times corresponding to the following compounds: dodecyl acetate (12 : OAc) (5.3),

14:OAc (85), Z5-14:OAc (24), Z7-14:OAc (76), (Z)-9-tetradecenyl acetate (Z9-14:OAc) (2.9), hexadecyl acetate (16:OAc) (4.1), (Z)-7-hexadecenyl acetate (Z7-16:OAc) (3.2), and (Z)-9-hexadecenyl acetate (Z9-16:OAc) (27). In a previous paper, moths from Dunedin were reported with a mean total of 13.2 ng of total Z5-14:OAc plus Z7-14:OAc per female (Foster et al., 1989). Analyses of extracts from the pooled sex pheromone glands of 10 female moths collected as larvae from Appleby Research orchard showed the same peaks as found in the Dunedin females, although the mean ratio of Z5-14:OAc to Z7-14:OAc was slightly different (35:65). All the above peaks, with the exception of the one corresponding to Z7-16:OAc, were observed in GC analyses of females from the Rukuhia colony. However, the mean ratio of Z5-14:OAc to Z7-14:OAc, 46:54, and the mean quantity of these two chemicals per female, 2.4 ng, were both significantly different from those observed in females from the other two areas as previously reported (Foster et al., 1989). Amounts of the other compounds in relation to the total amount of Z5-14:OAc and Z7-14:OAc were similar to those observed in the South Island females, although there was generally proportionately less Z9-16:OAc (ca. 15) in the Rukuhia colony females. Further GC analysis of the sex pheromone glands of females from all three areas using the Carbowax 20 M column, supported the above assignments.

GC-MS analysis of sex pheromone gland extract from Rukuhia females and comparison of the spectrum from each of the peaks with the mass spectra of authentic samples of the above compounds confirmed the above assignments (except for the peak corresponding to Z7-16:OAc, which was not detected in the Rukuhia females).

For the field-trapping trial, a blend consisting of all the identified compounds (including Z7-16:OAc) at different loadings (100, 300, and 1000 μg of total amount of Z5-14:OAc + Z7-14:OAc) was tested, as well as several blends with some of the less abundant compounds subtracted. As the trial was conducted near Appleby Research Orchard, the ratios of the various compounds in the blends tested were closer to the ratios found in the females analyzed from this area rather than the ratios found in females from Rukuhia. The results of the trial are shown in Table 1. There was no significant difference ($P > 0.05$) in catches between the blend (at 300 μg) containing 14:OAc, Z5-14:OAc, Z7-14:OAc, Z9-14:OAc, and Z9-16:OAc and the two blends (also at 300 μg) containing all these compounds and either 12:OAc and 16:OAc, or 12:OAc, 16:OAc, and Z7-16:OAc. Likewise, the catches to the eight-component blend were not significantly different ($P > 0.05$) at dosages of 100, 300, and 1000 μg.

Preliminary dose–response tests in the wind tunnel showed that male *P. excessana* flew upwind to the five component blend (at a dosage of 100 μg total Z5-14:OAc + Z7-14:OAc) used in the field-trapping trial. The first experiment (Figure 1A) compared the resp[illegible]s of males to the five-component blend

TABLE 1. TRAP CATCHES[a] OF MALE *Planotortrix excessana* TO VARIOUS BLENDS

Blend	Dosage[b] (μg)	Males caught/trap
C[c]	300	21.0 a[d]
C-16:OAc, -12:OAc	300	14.7 a
C-16:OAc -12:OAc -Z7-16:OAc	300	23.3 a
C	100	16.3 a
C	1000	19.0 a
Blank		0.0 b

[a] Field trial conducted at Lower Moutere, between October 10, 1988, and January 5, 1989. Trial consisted of three replicates. Insects were counted and removed, and traps rerandomized, each week.

[b] Total amount of Z5-14:OAc and Z7-14:OAc.

[c] C = 12:OAc (relative amount, 9), 14:OAc (100), 16:OAc (9), Z5-14:OAc (40), Z7-14:OAc (60), Z9-14:OAc (3), Z7-16:OAc (3), Z9-16:OAc (30).

[d] Means accompanied by the same letter are not significantly different at $P < 0.05$ (Duncan's multiple-range test).

versus blends with a single component removed. A high percentage of males (78–86%) oriented to all blends containing both Z5-14:OAc and Z7-14:OAc. In contrast, few (17%) of the males oriented towards the blend lacking Z7-14:OAc, and only 2% (1/59) oriented to the blend lacking Z5-14:OAc (both significantly less, $P < 0.01$, than to the other blends). The greatest percentage of males landing at the source was to the blend containing only the four 14-carbon acetates, although this percentage was not significantly different ($P > 0.05$) from the percentages of males landing at the source to the five-component blend or the blend lacking Z9-14:OAc. While removal of Z9-16:OAc or Z9-14:OAc from the five-component blend did not significantly affect the orientation or landing responses of males in the wind tunnel, removal of 14:OAc yielded a significantly lower ($P < 0.01$) landing response than to the four 14-carbon acetates (but not significantly different, $P > 0.05$, from the percentages of males landing at the source containing all five compounds or the source lacking Z9-14:OAc), suggesting that 14:OAc is a pheromone component of this species. The lowest percentages of males landing at the source were observed to the blends lacking either Z7-14:OAc (5% of males landed) or Z5-14:OAc (0% of males landed). These percentages were significantly lower ($P < 0.01$) than the percentages of males landing at the source for all other blends tested

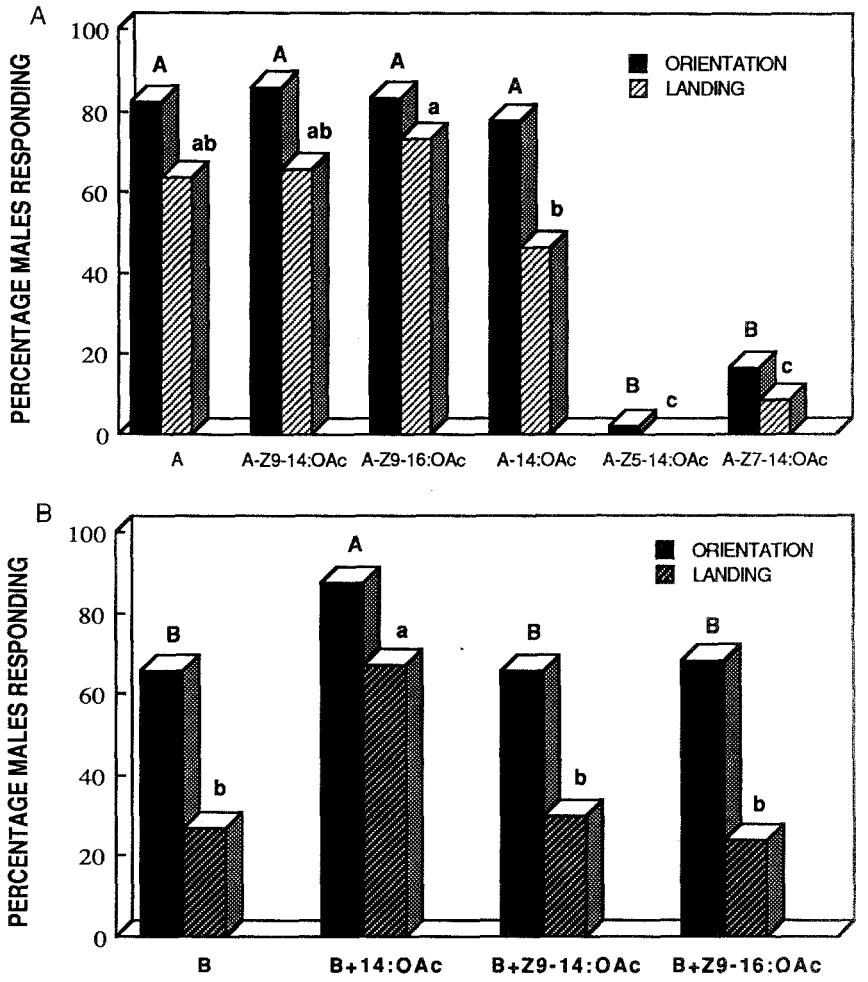


FIG. 1. Percentages of male *Planotortrix excessana* that oriented to or landed at the source to various blends. Bars with the same letters are not significantly different at $P < 0.01$ (Fisher's protected LSD). (A) "A" is a five component blend containing 40 μg (*Z*)-5-tetradecenyl acetate (*Z*5–14:OAc), 60 μg (*Z*)-7-tetradecenyl acetate (*Z*7–14:OAc), 100 μg tetradecyl acetate (14:OAc), 3 μg (*Z*)-9-tetradecenyl acetate (*Z*9–14:OAc), and 30 μg (*Z*)-9-hexadecenyl acetate (*Z*9–16:OAc). The other blends tested are all four component blends each with one of the five components of "A" removed. (B) "B" is a binary blend of 40 μg of (*Z*)-5-tetradecenyl acetate and 60 μg of (*Z*)-7-tetradecenyl acetate. The other blends tested are "B" with the addition of a single component (key to abbreviations and dosages as for (A).

in this experiment, and therefore both Z5–14:OAc and Z7–14:OAc are pheromone components of this species.

The second experiment compared the responses of males to a series of blends containing the addition of either 14:OAc, Z9–14:OAc, or Z9–16:OAc to a two-component blend of Z5–14:OAc and Z7–14:OAc (Figure 1B). The addition of 14:OAc to the two-component blend resulted in a significant increase ($P < 0.01$) in the percentage of males orienting towards the source relative to the two-component blend, clearly showing that 14:OAc elicits behavioral activity. However, the addition of either Z9–14:OAc or Z9–16:OAc to the two-component blend resulted in no significant ($P > 0.05$) increase in the percentages of males orienting towards the source over the two-component blend alone. These data were paralleled by the landing responses for which a significantly greater ($P < 0.01$) percentage of males landed at the source containing 14:OAc than for any of the other sources tested in this experiment. There were no significant differences ($P > 0.05$) in the percentages of males that landed at the source between the other three blends tested in this experiment.

Other Species. GC analyses of the *Planotortrix* "M" females showed, in addition to the two identified pheromone components, 14:OAc and Z5–14:OAc (Foster et al., 1987), a peak with retention times on both columns exactly matching that of Z7–14:OAc. The area of this peak was approximately 8% of the area of the Z5–14:OAc peak. A field trial comparing the standard two-component blend for this species (100 μg 14:OAc + 100 μg Z5–14:OAc) with a blend containing these two components plus 5 μg of Z7–14:OAc, showed that significantly higher ($P < 0.05$) numbers of males were caught in traps baited with the blend containing 5 μg of Z7–14:OAc (38 males caught) than in traps baited with the binary blend (nine males caught). The number of males caught in traps baited with the binary blend was not significantly different ($P > 0.05$) from the number of males caught in the blank traps (0 males caught).

Based on analyses of pooled sex pheromone glands from each species, ratios of Z5–14:OAc and Z7–14:OAc had been determined for *Planotortrix* "MBS" and *C. servana* in the respective ratios of approximately 3:97 and 33:67 (Foster and Dugdale, 1988). To establish the degree of variability, we analyzed the ratios of these two components in individual sex pheromone glands of *Planotortrix* "MBS" and *C. servana*. In *Planotortrix* "MBS" females ($N = 15$), the ratio of these two components was found to vary relatively little, between 1.4:98.6 and 8.0:92.0 between individual females, with a median of 3.1:96.9 (Figure 2A). A greater degree of variation in this ratio between individual females was found in *C. servana* females ($N = 35$), from 18:82 to 36:64 with a median of 27:73 (Figure 2A). For comparison, the ratios of these two components in individual female *P. excessana* from Waikato and Dunedin colonies are given in Figure 2B (from Foster et al., 1989).

Reinvestigation of the sex pheromone gland content of female *C. obli-*

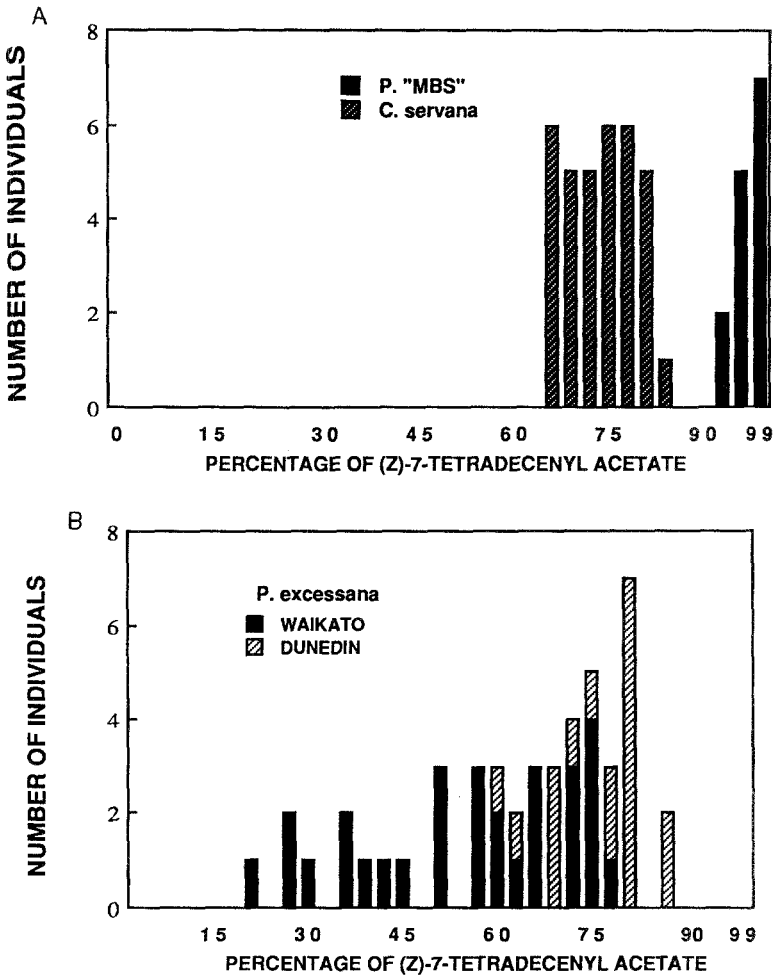


FIG. 2. Bar graph showing distributions of percentages of (*Z*)-7-tetradecenyl acetate [in total (*Z*)-7- and (*Z*)-5-tetradecenyl acetates] in pheromone glands of individual (A) *Planotortrix* "MBS" and *Ctenopseustis servana*, and (B) *Planotortrix excessana* from laboratory colonies established from insects collected from Rukuhia (Waikato) and Dunedin (from Foster et al., 1989).

quana (from Lincoln) and *P. notophaea* gave contrasting results. Using the SP2340 column (which allowed baseline separation of Z5–14 : OAc and Z7–14 : OAc), a small amount (approximately 2–3% of the amount of the previously reported pheromone component, Z5–14 : OAc) (Foster and Roelofs, 1987) of a compound with a retention time corresponding to that of Z7–14 : OAc was found

in the pooled sex pheromone glands of 20 female *C. obliquana* (Lincoln). However, in *P. notophaea*, which has Z7–14 : OAc as the most abundant long-chain acetate in its sex pheromone gland (Foster and Dugdale, 1988), no Z5–14 : OAc was detected in the pooled glands of 25 females (detection limit $< 1\%$ of amount of Z7–14 : OAc).

Extracts of the sex pheromone glands of two *P. flammea* females were analyzed by GC using the SP2340 column. In both females, compounds with retention times corresponding to Z5–14 : OAc and Z7–14 : OAc were found. The ratios (and quantities) of these two compounds, respectively, in the two gland extracts were 52 : 48 (33.9 ng) and 61 : 39 (13.8 ng). Small quantities (1–2 ng) of compounds with retention times matching those of dodecyl acetate and 14 : OAc were also found in both females.

DISCUSSION

Our chemical studies on the sex pheromone of a *P. excessana* sibling species have confirmed the tentative identification of Z5–14 : OAc and Z7–14 : OAc in female glands made by Galbreath et al. (1985). In addition, an additional six compounds (including Z7–16 : OAc), three saturated and three unsaturated aliphatic acetates, have been identified. The bioassay data show that of the eight compounds identified (including Z7–16 : OAc) in the sex pheromone gland of female *P. excessana* only three (Z5–14 : OAc, Z7–14 : OAc, and 14 : OAc) can yet be considered as components of the sex pheromone. When any of the other five compounds was added (alone or in combination) to blends of the three biologically active components no significant increase or decrease in the responses of males was observed. Whether these five additional chemicals are actually released by female *P. excessana* is not known at this time. In this study, only a limited number of combinations of all the chemicals and only one ratio of Z5–14 : OAc to Z7–14 : OAc were tested. Given the very wide range of ratios of Z5–14 : OAc to Z7–14 : OAc produced by females, and the differences between populations (Foster et al., 1989), more extensive testing of males of various populations to different ratios and combinations of chemicals is warranted. Such a study is in progress.

The *P. excessana* sibling species studied herein is found extensively throughout New Zealand often in sympatry with the other *P. excessana* sibling species (utilizing Z8–14 : OAc and 14 : OAc as components of its sex pheromone) and the two sibling species of *C. obliquana* (Foster et al., 1986; Foster, unpublished). These "two" described species, along with the introduced Australian tortricid, the lightbrown apple moth, *Epiphyas postvittana*, are considered major pests of important fruit crops in New Zealand such as apple and kiwifruit (Wearing et al., 1990). The identification of an attractive blend of

components for this *P. excessana* sibling species means that all five of these species of leafroller moths can now be monitored in commercial orchards using specific sex pheromone-baited traps. This should facilitate the determination of the relative pest status of each of the sibling species on different crops in different areas around New Zealand.

This study has shown that the components Z5–14 : OAc and Z7–14 : OAc occur in other species of *Planotortrix* and also in two species of *Ctenopseustis*. While both Z5–14 : OAc and Z7–14 : OAc had been found previously in *Planotortrix* "MBS" and *C. servana* females (Foster and Dugdale, 1988), Z7–14 : OAc had not been identified previously in the sex pheromone glands of female *Planotortrix* "M" or the *C. obliquana* sibling species (Foster et al., 1987; Foster and Roelofs, 1987). In the case of *Planotortrix* "M" the addition of Z7–14 : OAc to the reported pheromone blend of Z5–14 : OAc and 14 : OAc resulted in a significant increase in the numbers of males caught in traps baited with this tertiary blend over traps baited with the binary blend, showing that Z7–14 : OAc is a pheromone component of this species. The small number of males caught in this trial parallels the small number of larvae of this species collected for analysis and possibly indicates a decrease in the population size in this particular area of the mangrove swamp in the Firth of Thames since the last field trapping was conducted there in 1984. Further support for this is the small number of males (not significantly different from zero) caught in traps baited with the binary blend in this study, whereas previously traps baited with this blend had yielded reasonably high catches of males of this species (Foster et al., 1987).

Components Z5–14 : OAc and Z7–14 : OAc also have been found in some Australian tortricid species in the tribe Epitymbiini (Horak et al., 1988). It appears, therefore, in these Australian species and the New Zealand species investigated here that these two compounds are utilized in combination in pheromonal communication as are the geometric isomers (*Z*)-11-tetradecenyl acetate (Z11–14 : OAc) and (*E*)-11-tetradecenyl acetate (*E*11–14 : OAc) in many species of Oriental, New Zealand, and Holarctic Tortricidae. One of the most intensively studied groups of Holarctic Tortricidae is the guild of species feeding on apples in New York State. Species in this guild are apparently sympatric and have similar mating rhythms but have specific communication using discrete ratios of the two geometric isomers along with other components (Roelofs and Brown, 1982).

While the use of discrete ratios of Z5–14 : OAc and Z7–14 : OAc in these New Zealand and Australian species (Figure 3) largely parallels the use of ratios of the geometric isomers Z11–14 : OAc and *E*11–14 : OAc by the New York tortricid species, there is one clear difference between the two pheromone systems: the great degree of intraspecific variation in ratios of the two compounds exhibited by *P. excessana* (Figures 2B and 3; see also Foster et al., 1989). The

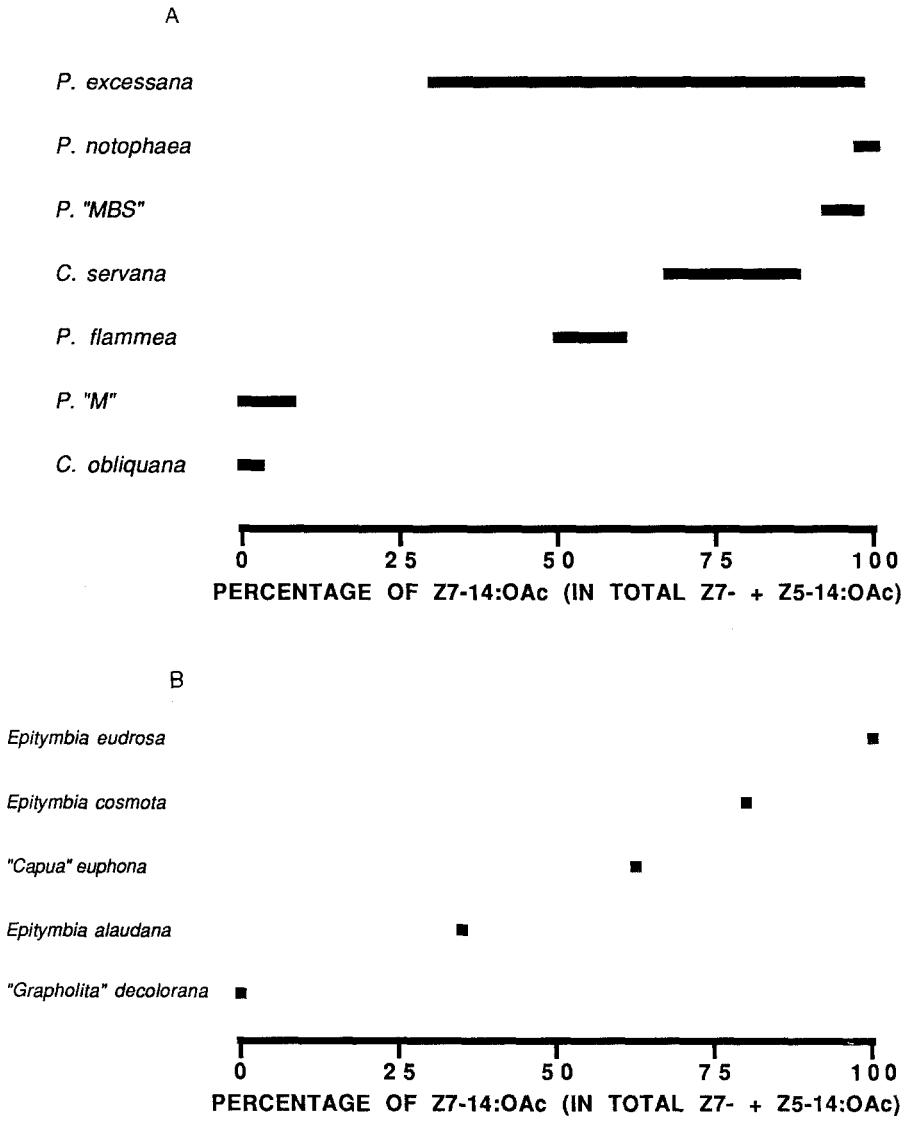


FIG. 3. Range of percentages of (Z)-7-tetradecenyl acetate [in total (Z)-7- and (Z)-5-tetradecenyl acetates] in female sex pheromone glands, obtained from gas chromatographic analyses of: (A) seven species in the New Zealand genera *Planotortrix* and *Ctenopseustis*; *P. excessana* data from Foster et al. (1989); *P. notophaea* data from Foster and Dugdale, (1988); (B) five Australian species of Tortricidae in the tribe Epitymbiini; data from Horak et al. (1988).

ratios in both pheromone systems are determined during the biosynthesis of the respective chemicals. The biosynthetic pathways for *E*- and *Z*11–14:OAc in the New York tortricids have been extensively studied (Roelofs and Wolf, 1988). Both pathways are intimately linked, with production of the putative precursors of the two geometric isomers occurring by Δ11-desaturation; the final ratio of the two pheromone components is determined during the subsequent reduction and acetylation stages (Roelofs and Bjostad, 1984). Roelofs and Bjostad (1984) proposed that *Z*5–14:OAc and *Z*7–14:OAc are biosynthesized along similar pathways, respectively, from the ubiquitous palmitate and oleate moieties by two-carbon chain-shortening. If this is the case, then the similarity of these pathways utilizing these moieties may explain why *Z*5–14:OAc and *Z*7–14:OAc are usually found together in the above closely related species. However, clearly these pathways are not so intimately linked nor are the precursors so closely related as are the two geometric isomers (*E*- and *Z*11–14:OAc); therefore, we might intuitively expect the ratio of *Z*5–14:OAc to *Z*7–14:OAc to be more variable within a species, as we observed in *P. excessana*. Nevertheless, we found the ratios of these two chemicals to be tightly regulated within a species, as in *P.* "MBS." The close relatedness of the species used in this study and the differences in regulation of the ratio of the two components make these species a useful group of insects for studying intraspecific variability in production and response of pheromonal sexual communication.

Between individual *P. excessana* females, ratios of the two components vary from 3:97 to 71:29, clearly overlapping with the ratios produced by females of *Planotortrix* "MBS," *C. servana*, and *P. flammea*. It is likely that other chemicals (such as 14:OAc in *P. excessana*) contribute to the respective specificity of the communication systems of these species, although differences in temporal behavioral activity patterns and spatial distributions cannot be ruled out. Although *Planotortrix* "MBS," *P. flammea*, and *C. servana* have limited distributions, all are locally sympatric with *P. excessana* (Dugdale, personal communication). *P. flammea* and *Planotortrix* "MBS" are host restricted to *Hebe* species (Scrophulariaceae), and *Olearia* and *Brachyglottis* species (Asteraceae), respectively, but *C. servana* is polyphagous with many hosts in common with *P. excessana* (Green and Dugdale, 1982). It is hoped further study on the ecology and sex pheromones of these species will elucidate the factors involved in the specificity of their respective communication systems.

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CHEMICAL STUDIES OF RECTAL GLAND SECRETIONS OF SOME SPECIES OF *Bactrocera dorsalis* COMPLEX OF FRUIT FLIES (DIPTERA: TEPHRITIDAE)

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Abstract—Male-produced rectal gland secretions of a number of species now considered to be members of the *Bactrocera dorsalis* complex of fruit flies have been examined by combined gas chromatography–mass spectrometry. The structures of a number of previously unreported compounds have been confirmed by synthesis and chromatographic comparisons. Compounds identified in these secretions include fatty acids, esters, amides, keto alcohols, diols and spiroacetals. These chemical findings are in agreement with taxonomic revisions based on other criteria.

Key Words—*Bactrocera dorsalis, Bactrocera occipitalis*, Diptera, Tephritidae, male pheromone, spiroacetal, ketoalcohol.

INTRODUCTION

The *dorsalis* complex of fruit flies comprises a large number of species bearing a taxonomic resemblance to *Bactrocera*[4] *dorsalis* (Hendel), and many are major pests of a wide variety of fruit and vegetable crops. Members of the complex extend throughout Asia, the Oceanian region, and into Australia. The taxonomy

[4] *Bactrocera* is the new genus name applied, in the general taxanomical revision (Drew, 1989), to a large number of fruit fly species previously classified in the *Dacus* genus.

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of this complex is being reviewed (Drew, unpublished), and the differentiation of close species has been greatly facilitated by techniques such as electron microscopy and tissue enzyme analysis, in addition to morphological and biological data. In a number of cases, species within the complex have been incorrectly identified. Consequently, distribution and host records have been wrongly attributed, and some species declared as pests have no economic significance and more restricted distributions.

B. dorsalis, a member of the complex, is restricted in habitat from India to southern China, Taiwan, and Hawaii, and is not as widespread as previously reported (Drew et al., 1982). *B. occipitalis* (Bezzi) was originally described from the Philippines and later recorded as widespread over Southeast Asia (Hardy, 1974). It is, in fact, confined to the Philippines. Three sibling species, in the past confused with *B. dorsalis* or *B. occipitalis*, occur in the Philippines and Malaysia, but these species have not, as yet, been assigned scientific names. These will be referred to as Malaysian A (Mal A), Malaysian B (Mal B), and Philippine B (Phil B). Mal B and Phil B closely resemble *B. dorsalis*, while Mal A resembles *B. occipitalis*. The major purpose of this work was to characterize chemically the volatile components of the glandular secretions of these species and thus possibly support any taxonomic revisions, as well as to extend the pheromone profile of these economically important Tephritidae.

METHODS AND MATERIALS

Bactrocera dorsalis specimens from the USDA culture, Honolulu, Hawaii, were air-freighted to Brisbane in Dry Ice. Mal A, Mal B, and Phil B specimens were trapped in the field in Malaysia and the Philippines, using male lure traps. The traps were designed to prevent the attracted flies from feeding on the lures and to maintain the flies in a live condition until freezing. The flies were snap-frozen and stored in an ultra-low deep freeze (−80°C) and transported in Dry Ice to Australia and stored at −80°C until dissection.

Gland Secretion Analysis and Identification

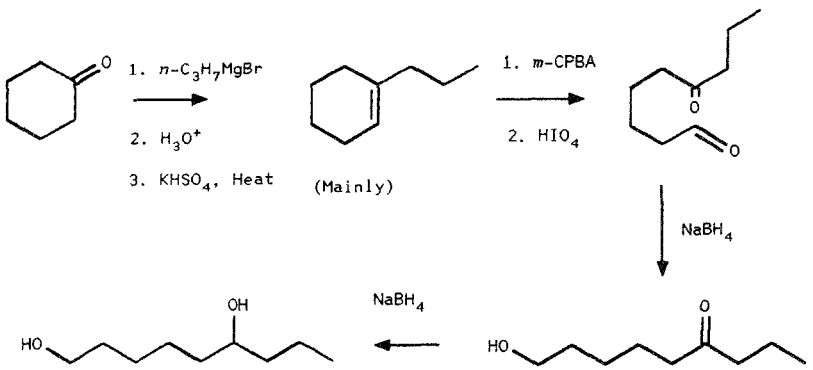
The rectal glands of sexually mature adult male flies were excised and extracted into acetone and/or pentane as described earlier (Kitching et al., 1989). Combined GC-MS examinations were performed using a Finnegan Mat 1020 or Hewlett Packard HP5992 instrument, employing a nonpolar column. Low-resolution mass spectra refer to combined GC-MS measurements, and high-resolution mass spectra were recorded on a Kratos MS-25RFA spectrometer.

Proton magnetic resonance spectra were recorded at 400 MHz (FT mode) on a JEOL JNM-GX400 spectrometer, or in some cases at 60 MHz (CW mode) on a JEOL PS100 spectrometer. Deuteriochloroform was employed as solvent

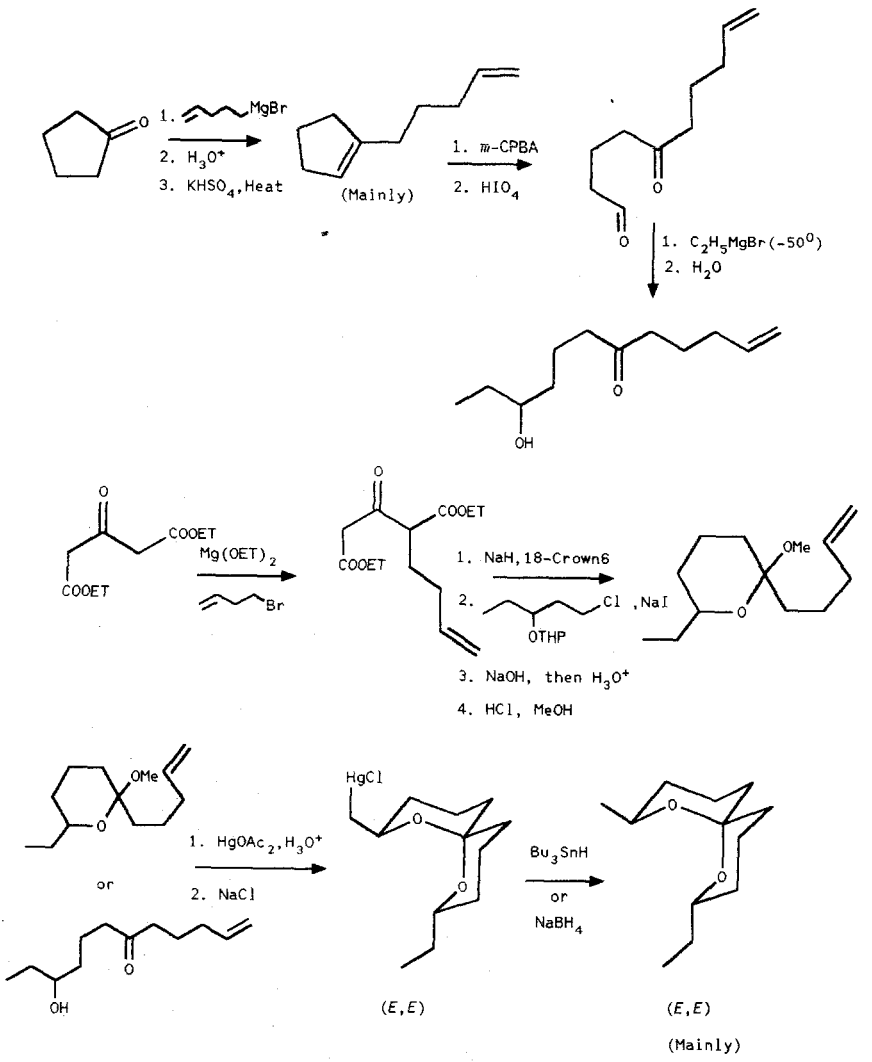
and chemical shifts (δ values) are relative to internal tetramethyl silane (0.0 ppm) or residual $CHCl_3$ (δ 7.24). [^{13}C]NMR spectra were recorded at 100 MHz, utilizing $CDCl_3$ as solvent, and chemical shifts are relative to the central component of the $CDCl_3$ triplet at 77.00 ppm.

Synthesis of Certain Glandular Components (Schemes 1 and 2)

6-Oxo-1-nonanol. The Grignard reagent, from *n*-propyl bromide (21.4 g) and magnesium turnings (4 g) in ether (50 ml), was cooled to 0°C, and cyclohexanone (17 g) in ether (50 ml) was added at such a rate that the temperature did not rise above 5°C. This mixture was stirred overnight and then poured onto ice, and dilute sulfuric acid (15%) was added to dissolve the magnesium salts. The ether layer was separated, and the aqueous layer was extracted with ether (2 × 50 ml), and the combined ether fractions were dried ($MgSO_4$) and evaporated to yield an oil. This was fractionally distilled to provide the required tertiary alcohol, 6.5 g (bp 76–82/12 mm). [^{13}C]NMR: 14.5, 16.0, 22.1 (2C), 25.7, 37.2 (2C), 44.7, 71.2. This alcohol (6.5 g) was placed in a distillation flask with finely powdered $KHSO_4$ (6.5 g), and the mixture was stirred and heated at 120°C for 30 min followed by 30 min of heating at 160–170°C. During this time, water and the product were distilled, with the latter purified by passing down a short (2 × 1-in.) silica column and eluting with hexane. The alkene obtained was a 4 : 1 mixture of the desired 1-propyl-1-cyclohexene (*endo*-dehydration) and the alternative *exo*-alkene. These alkenes were characterized by their [^{13}C]NMR and mass spectra. 1-Propyl-1-cyclohexene: [^{13}C]NMR: 13.8, 20.9, 22.7, 23.1, 25.3, 28.3, 40.3, 120.7, 137.8. Mass spectrum: M^+ 124 (34), 95 (33), 82 (62), 81 (100), 79 (26), 77 (13), 68 19), 67 (63), 65 (10). *exo*-Alkene: 14.8, 20.4, 27.1, 27.9, 28.6, 28.8, 37.2, 123.2, 138.9. Mass spectrum: M^+ 124 (34), 95 (32), 82 (62), 81 (74), 79 (22), 77 (12), 68 (24), 67 (100).



SCHEME 1. Synthesis of 6-oxo-1-nonanol and 1,6-nonandiol.



SCHEME 2. Synthesis of 2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane.

The above alkene mixture (4:1, 0.5 g) was dissolved in dichloromethane (50 ml) and *m*-chloroperbenzoic acid (1.2 g) was added, while stirring with 0.5 M sodium bicarbonate solution (20 ml). After ca. 3 hr, the layers were separated and washed with 10% KOH solution (2 × 20 ml) and then H_2O (4 × 20 ml). The dried ($MgSO_4$) organic layer was evaporated to yield a clear oil. These mixed epoxides (2.0 g) were not purified but were added to a solution of periodic acid (4.88 g) in dry ether (400 ml), and after ca. 1 hr, the desired ketoal-

dehyde, 6-oxononanal, was the predominant product (>80% by GC). The filtered reaction solution was washed with H_2O, 10% $NaHCO_3$, H_2O again, and then dried and evaporated. The desired 6-oxononanal showed the aldehydic proton in the [^{1}H]NMR spectrum at δ 9.80 (narrow triplet). [^{13}C]NMR: 13.7 (CH_3), 17.3, 21.6, 23.1, 42.3, 43.7, 44.8, 202.2 (CHO) and 210.7 (C=O). Mass spectrum: M^+ 156 (4), 128 (8), 113 (15), 112 (7), 99 (8), 95 (23), 87 (5), 86 (19), 85 (17), 71 (76), 67 (55), 58 (24), 57 (18), 55 (15), 43 (100). Very careful reduction with $NaBH_4$ in ethanol (~0°C) provided 6-oxo-1-nonanol as a clear oil with very slight contamination with 1,6-nonanediol from reduction of both carbonyl functions. 6-Oxo-1-nonanol: Mass spectrum: M^+ 158 (2), 140 (5), 115 (14), 112 (5), 99 (6), 97 (30), 86 (32), 79 (8), 73 (12), 71 (68), 70 (9), 69 (58), 67 (6), 58 (48), 57 (10), 55 (36), 43 (100). [^{13}C]NMR: 13.7, 17.3, 23.4, 25.4, 32.4, 42.7, 44.8, 62.5, 211.8. [^{1}H]NMR: 0.84 (3H, t of d, J = 7.3 Hz, 1.7 Hz, CH_3), 1.29–2.35 (12H, m), 2.56 (1H, br OH), 3.56 (2H, t of d, J = 6.6, 2.0 Hz, $\underline{CH_2}OH$). [This compound correlated well (0.994) with the natural product on mass spectral comparison.]

1,6-Nonanediol resulted from reduction of both carbonyl functions of the intermediate 6-oxononanal. Mass spectrum: M^+ 160 (0), 117 (14), 99 (34), 81 (64), 73 (41), 71 (125), 70 (19), 57 (37), 55 (100). [^{13}C]NMR: 14.1, 18.8, 25.4, 25.8, 32.6, 37.3, 39.7, 62.5, 71.5. [^{1}H]NMR: 0.92 (3H, t, 7 Hz, CH_3), 1.25–1.52 (10H, M), 1.58 (2H, m), 3.58 (5H, m including —OH).

The above synthesized samples of 6-oxo-1-nonanol and 1,6-nonane-diol were spectroscopically identical with samples synthesized by a completely different route (O'Shea and Kitching, 1989).

2-Ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane. 5-Oxo-9-decenal was acquired in a manner analogous to that described above for 6-oxo-1-nonanol except that cyclopentanone was reacted with the Grignard reagent from 5-bromo-1-pentene in the first step. The details of this synthesis have been described elsewhere (Nishiyama et al., 1989). The Grignard reagent (ether) from ethylbromide (0.55 g) was added dropwise to a solution of 5-oxo-9-decenal (~1 g) in ether at −50°C and the solution allowed to warm slowly (3 hr) to room temperature. The reaction mixture was poured onto ice and 5% H_2SO_4 was added to dissolve salts. The ether layer was separated, washed (with H_2O), dried, and evaporated. GC-MS examination showed two major components, the first eluting being unreacted ketoaldehyde and a longer-retention-time component appearing as a broad peak. Chromatography on silica (hexane–ether, 3:1) provided fractions rich (~80%) in the presumed unsaturated ketoalcohol resulting from predominant Grignard addition to the aldehyde function. That selective Grignard addition to the aldehyde carbonyl had occurred was shown by the [^{13}C]NMR spectrum of the purified alcohol, which retained the ketonic carbonyl at 210.6 ppm and revealed a signal in the [^{1}H]NMR spectrum at δ 3.60 (1H, quintet), appropriate for the desired 7-oxo-11-dodecene-3-ol.

[^{1}H]NMR: δ 1.0 (3H, t, J = 7 Hz, CH_3), 1.1–2.7 (15H, m, CH_2). 3.60 (1H, q, J = 7 Hz, > CHOH), 5.1 (2H, m), 5.8 (1H, m). (This sample was contaminated slightly with unreacted ketoaldehyde). [^{13}C]NMR: 9.9, 19.7, 22.8, 30.2, 33.1, 36.3, 41.9, 42.6, 72.8, 115.4, 137.7, 210.6. (These chemical shifts are consistent with the overwhelming predominance of the open chain hydroxyketone form). Mass spectrum: M^+ 198 (0), 180 (13), 126 (49), 111 (13), 97 (34), 84 (33), 83 (30), 81 (10), 79 (10), 70 (18), 69 (34), 68 (60), 67 (16),.57 (18), 55 (100).

Alternative Synthesis of 7-Oxo-11-dodecene-3-ol. Diethyl 3-oxo-glutarate was alkylated with 4-bromo-1-butene in the manner described (Naoshima et al., 1984) to yield diethyl-2-(but-3′-ene)-3-oxoglutarate in ca. 75% yield after chromatography on silica, eluting with hexane–ether, 4 : 1. Mass spectrum: M^+ 256 (0), 211 ($M - C_2H_5$, 3), 156 (36), 128 (31), 123 (15), 101 (22), 95 (14), 73 (20), 55 (40), 29 (100). [^{13}C]NMR: 14.1 (2C), 27.0, 31.3, 48.4, 58.0, 61.5, 61.6, 116.0, 137.0, 166.7, 169.1, 197.5. [^{1}H]NMR: 1.2 (6H, t, J = 7 Hz, CH_3), 1.9–2.1 (4H, m), 3.5–3.7 (3H, m), 4.1 (4H, m, CH_2), 5.0 (2H, m), 5.7 (1H, m). This diester (4 g) was dissolved in dry toluene (30 ml) and added dropwise to a suspension of sodium hydride (50% mineral oil dispersion, 0.77 g) and 18-crown-6 (0.4 g) in dry toluene (50 ml) with constant stirring at room temperature for about 1 hr. To this solution was added the tetrahydropyranyl ether of 1-chloro-3-pentanol (4 g) [from $NaBH_4$ reduction of commercially available (Aldrich) 1-chloro-3-pentanone] and finely powdered NaI (2 g), after which the mixture was stirred and refluxed for 15 hr (GC monitoring). The solvent was removed by vacuum distillation and the residue heated with 15% aqueous NaOH (100 ml) for 20 hr. The cooled hydrolyzed mixture was acidified with concentrated HCl, and the solution saturated with salt and extracted with ether. The dried ether solution was evaporated to provide a brown liquid, which was dissolved in methanol (80 ml) and to which were added concentrated HCl (two drops) and H_2O (1 ml). This solution was filtered and evaporated to leave a brown liquid that was distilled (60°C/1 mm) to provide 1.5 g of a colorless oil, which was the methoxy acetal of 7-oxo-11-dodecan-3-ol on the basis of the ^{13}C signal at 99.3 pm and the lack of carbonyl absorption. [^{13}C]NMR: 10.0, 19.0, 22.7, 29.1, 30.5, 32.6, 33.9, 36.1, 47.1, 71.4, 99.3, 114.6, 138.6.

Both forms of the 7-oxo-11-dodecen-3-ol (i.e., as open hydroxyketone or cyclic methoxy acetal) were subjected to Hg(II) cyclization to generate the 1,7-dioxaspiro[5.5]undecane skeleton. Thus the methoxyacetal (0.5 g) was added to a stirred solution (room temperature) of $Hg(OAc)_2$ (1.5 g) in THF (30 ml) and 1% aqueous $HClO_4$ (30 ml). After 30 min (during which time a white precipitate formed), sodium chloride (excess) was added (to form the chloromercury derivative) and the solution extracted with CH_2Cl_2 (3 × 100 ml). This organic phase was washed with water (50 ml) dried ($MgSO_4$), and evaporated without heat. A white solid (0.75 g, 70%), mp 105°C (colorless needles), was

obtained and presumed to be the chloromercurial. This was confirmed by the mass spectrum and [^{13}C]- and [^{1}H]NMR spectra. Accurate mass: Calcd. for $C_{12}H_{21}O_2{}^{202}Hg^{35}Cl$ = 434.0936. Observed 434.0932. The [^{13}C]NMR spectrum showed that predominantly one diastereomer of 2-(chloromercuri)methyl-8-ethyl-1,7-dioxaspiro[5.5]undecane had formed, and this was clearly the *E,E*-isomer on the basis of comparisons of its [^{13}C]NMR shifts with those of similar 2,8-dialkyl substituted spiroacetals of known relative configurations (Kitching et al., 1989). [^{13}C]NMR: 10.2, 19.0, 19.1, 29.3, 30.8, 35.1 (2C), 35.4, 39.5, 68.2, 71.3, 96.8. (Lower intensity signals probably correspond to the two possible *E,Z* diastereomers). [^{1}H]NMR: 0.9 (3H, t, J = 7 Hz, CH_3), 0.9–2.5 (m, ~16H), 3.3 (1H, m), 3.9 (1H, m).

The above mercurial (150 mg), dissolved in benzene (1 ml) was treated with Bu_3SnH (1 ml) and a small crystal of AIBN at room temperature. After 15 min, the solution was filtered through celite to remove Hg residues, etc., and washed with CH_2Cl_2. This was stirred with aqueous KF (10%), separated, and evaporated to provide 2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane very predominantly as the *E,E* diastereomer. Demercuration with sodium borohydride also has been employed and avoids some purification problems that arise when Bu_3SnH is used. A pure sample of this spiroacetal mixture was readily obtained as an oil by preparative gas chromatography on a nonpolar column. GC-MS examination revealed the presence of two diastereomers of the spiroacetal, which exhibit very similar mass spectra. Mass spectrum (*E, E*): M^+ 198(5), 169(6), 129(21), 126(17), 125(9), 115(41), 114(11), 112(38), 111(15), 97(26), 84(14), 83(27), 69(38), 55(72), 43(66), 42(39), 41(100). The spectral properties agree completely with those of a sample subsequently obtained by another route (O'Shea and Kitching, 1989), and full NMR spectral details are reported there. Entantioselective synthesis of the isomers of 2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane have been completed recently (Perkins et al., 1990).

RESULTS AND DISCUSSION

GC-MS analysis of the acetone extract of the rectal gland of sexually mature males of *B. dorsalis* indicated that the major components were long-chain fatty acids, with one as the ethyl ester. A number of minor compounds of higher volatility were also present. Treatment of the extract with diazomethane resulted in the observation of the methyl esters of the fatty acids shown in Figure 1, and of these new compounds the major one was the trimethyl ester of citric acid (**1**), along with the trimethyl ester of phosphoric acid (**2**) and dimethylsuccinate (**3**).

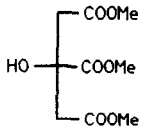
The more volatile very minor components were of interest with one showing an apparent M = 198 (5%) and loss of 29 and 15 mu, corresponding to

FATTY ACIDS: C_{12}, C_{14}, C_{16},C_{18} (Saturated)

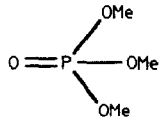
C_{16},C_{18} (Mono-unsaturated)

C_{12}-ethyl ester (Saturated)

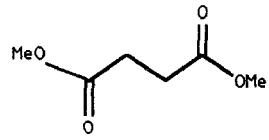
After methylation with CH_2N_2



(1)

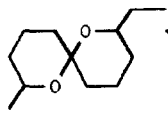


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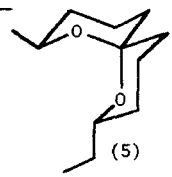


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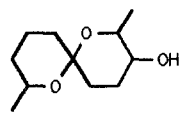
Minor volatile Components



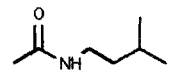
(4)



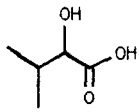
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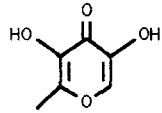


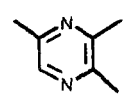
(6)



(7)







(14)

FIG. 1. Compounds observed by solvent extraction of adult male *Bactrocera dorsalis* (Hendel).

loss of C_2H_5 and CH_3, respectively. Intense ions at m/z = 112 and 115 and other features of the mass spectrum suggested the structure to be a 2,8-dialkyl-1,7-dioxaspiro[5.5]undecane, specifically 2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane (**4**), presumably as the more stable *E*, *E* diastereomer (**5**). This was confirmed by comparisons with the spectra and chromatographic properties of an independently synthesized sample and represents an example of the unusual class of even-carbon-numbered insect-derived spiroacetals. A low level

of 3-hydroxy-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (**6**) was observed also and confirmed by mass spectral comparisons with an authentic sample (Kitching et al., 1989). A full listing of compounds is shown in Figure 1.

A "smokelike" substance emitted by males of *B. dorsalis* at dusk (the mating period of these tephritids) has been reported (Ohinata et al., 1982) and ca. 90% of the smoke collected on Millipore filters was (tri)sodium phosphate, in agreement with our identification of the phosphate ester (from the acid). A minor component of the smoke was *N*-(2-methylbutyl)propanamide (Ohinata et al., 1982), which was absent from the glandular secretion. [*N*-(3-methylbutyl)acetamide (**7**) was a glandular component.]

The volatile secretions produced by female *B. dorsalis* from culture in Hawaii also have been studied (Baker and Bacon, 1985). The volatiles were collected on charcoal filters, and GC-MS analysis of the solvent extracts showed the presence of one major compound, *N*-(3-methylbutyl)acetamide (**7**) and two minor spiroacetals in addition to **5**. These were 2,8-dimethyl and 2-*n*-propyl-8-methyl derivatives, **8** and **9**, respectively, of the 1,7-dioxaspiro[5.5]undecane system. An homologous series of ethyl esters of the C_{12}, C_{14}, and C_{16} saturated fatty acids, as well as of the C_{16} unsaturated fatty acid were identified also. These compounds are shown in Figure 2.

Mal A is very similar to *B. occipitalis* and occurs in Malaysia only. This species was examined by us some years ago and aspects of the results were mentioned in a brief report (Kitching et al., 1986). These specimens were field

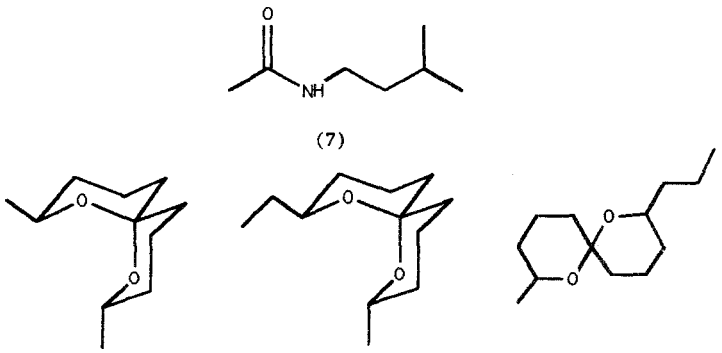


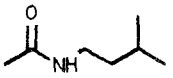
FIG. 2. Compounds observed in the volatiles of female *Bactrocera dorsalis* (Hendel) (Baker and Bacon, 1985).

collected in Malaysia and were incorrectly reported as being *Dacus occipitalis*. This species is now regarded (Drew, unpublished) as taxonomically distinct from *B. occipitalis*, but Drew's study will be reported in due course. We have been unable to obtain authentic *B. occipitalis* for analysis.

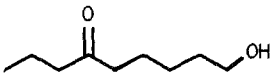
A more recent examination of this Mal A confirms the results obtained on earlier specimens, except for the absence of methyl eugenol metabolites that were detected in the earlier study. (Methyl eugenol is the lure used for trapping, but the trap design now used prevents feeding on the attractant). Temperature programmed gas chromatography (SE 54 columns) of the acetone extract showed the presence of one major (~70%; 5 μg/fly) and several minor components. This major component exhibited apparent M = 158 (2.4%) and (M − 18) at *m/z* = 140 (5%), suggesting an alcohol. Additional ions at *m/z* 71 (75%) and 115 (16%) indicated that 6-oxo-1-nonanol ($C_9H_{18}O_2$) (**10**) was a likely structure, and this was supported by the McLafferty ion at *m/z* = 86 (37%). Synthesis of the previously unreported 6-oxo-1-nonanol (**10**) was conducted as outlined in Methods and Materials, and identity with the natural product was established by mass spectral and gas chromatographic comparisons. A minor compound (ca. 4%) eluting slightly after 6-oxo-1-nonanol showed highest *m/z* = 117 (14%) with other ions at *m/z* = 99 (34%) (117–18), and *m/z* = 81 (64%). This information coupled, with the chromatographic data, suggested 1,6-nonanediol (**11**) was present, and this was confirmed by comparisons with an authentic sample acquired by reduction of the synthesized 6-oxo-1-nonanol. The EI mass spectrum of the diol lacks a molecular ion (M^+ = 160), but exhibits a prominent ion at *m/z* = 117, corresponding to $HO\overset{+}{C}H(CH_2)_5OH$. (M − C_3H_7). The chirality of this diol would be of some interest, but this has not yet been determined. *N*-(3-Methylbutyl)acetamide (6%) (**7**) and ethyl 3-methyl-2-hydroxy butanoate (12) (~5%) (0.97 match with library spectrum) were identified also. The spiroacetal, 2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane (**5**) and pyrazine (**13**) were very minor components, with the structure **13** being confirmed by mass spectral and gas chromatographic comparisons (retention time, coelution) with an authentic sample. Analysis of pentane extracts provided the same chemical profile. The compounds are displayed in Figure 3.

The analysis of the second species from Malaysia (Mal B) provided a vastly different result. The major components were the C_{16}, C_{18} saturated and unsaturated acids, together with high-molecular-weight hydrocarbons. In the volatile region, very low levels of trimethyl pyrazine (**14**), spiroacetals (**5** and **8**), and *N*-(3-methylbutyl)acetamide (**7**) were present in various analyses. This species has not been examined previously, but it is taxonomically very close to *B. dorsalis* itself (discussed above), and both are sparse in terms of volatile glandular components. Fatty acids are the major components in each species, and

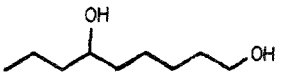
Major Components



(7)

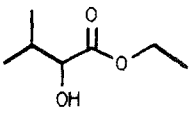


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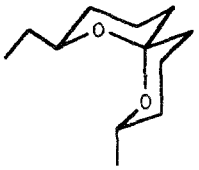


(11)

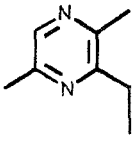
Others



(12)



(5)



(13)

FATTY ACIDS: C_{16}, C_{18} (Saturated)

C_{16}, C_{18} (Mono-unsaturated)

C_{18} (Di-unsaturated)

FIG. 3. Compounds observed by solvent extraction of male Mal A.

both secrete low levels of spiroacetal (**5**), trimethylpyrazine, and amide (**7**). There were differences, however, including the absence of the C_{12} ethyl ester and the appearance of spiroacetal (**8**) in Mal B.

The analysis of Phil B revealed a similarity to the dorsalis type, with a low level of volatile compounds, with fatty acids again being dominant. However, in comparison with Mal B, large amounts of C_{12} and C_{14} (saturated) and C_{16} (saturated and unsaturated) fatty acid ethyl esters were present. Small amounts of spiroacetal (**5**), amide (**7**), and trimethyl pyrazine were observed but not spiroacetal (**8**). Thus the analysis of Phil B is most similar to that of *B. dorsalis*, in which spiroacetal (**8**) was absent but the C_{12} fatty acid ethyl ester was present.

These results indicate that in Malaysia, Mal A and Mal B are two very closely related but distinct sibling species of the *dorsalis* complex. Although taxonomically very similar (being difficult to differentiate by normal morphological examination), these two species show vast differences in the chemistry of their rectal gland extracts. Mal A, in fact, has a glandular chemistry that is most distinct from that of any other species examined, whereas Mal B, Phil B, and *B. dorsalis* have similar glandular components. Minor, consistent differences were found, however, between Mal B, Phil B, and *B. dorsalis*. These results have important application in the taxonomic studies of this most destructive fruit fly complex and assist in defining the species. They also add to our knowledge of the general chemistry of fruit flies.

Acknowledgments—The authors are grateful to Dr. Sabine Krohn for an authentic sample of pyrazine (**13**).

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Letter to the Editor

DE GEER'S PIONEERING PHYTOCHEMICAL OBSERVATION

Chemical ecology is thriving on many fronts, making history. Yet there is little in contemporary writing to remind us of the unfolding of the discipline, of early events and discoveries that might have been real "firsts" in the field, but have faded into obscurity. Sometimes cited is the paper by Wray (1690), on formic acid production in ants, doubtless one of the first publications on the chemical defenses of arthropods, but there must be other early citations awaiting rediscovery. I recently chanced upon some writings of the Swedish pioneer entomologist Karl De Geer (1720–1788), in which some chemical-ecological questions are raised that are remarkably modern in flavor.

In his seven-volume treatise on insects, De Geer (1776) provides mostly descriptions and commentary, as was typical for the time, but he also gives accounts of genuine experiments. These are often "spur of the moment" in design, but they are presented lucidly, conceived rationally, and controlled. In Volume 1, Section 3, beginning on page 58, he provides a delightful account of a small moth, already noted by two famous contemporaries (Frisch, 1720–1738; Rösel, 1746), which, as a larva, lives on pine, within a "gall" made up of resin from the host. The moth is almost certainly *Petrova* (formerly *Evetria*) *resinella* (L.), a tortricid, nowadays mentioned routinely in treatises (for example, Lampert, 1907).

De Geer describes in some detail how the larva feeds by carving out a groove along the length of a small branch of its host, and how the surrounding gall, which takes the form of an igloolike enclosure, is formed by the resin that oozes from the plant wound (Figures 1 and 2). He notes that the larva lives protectively confined within the gall and that it encrusts the enclosure from within with some of its feces. Not surprisingly, given that the difference between the vascular and resin systems of plants had not been clarified at the time, he errs in his interpretation of the nature of resin, which he takes to be part of the plant's nutrient juices ("Nahrungssafte des Baums")[1]. However, he does betray

[1] De Geer's treatise originally appeared in French. I am here quoting from the German edition (De Geer, 1776), where the phraseology and spelling are sometimes archaic.

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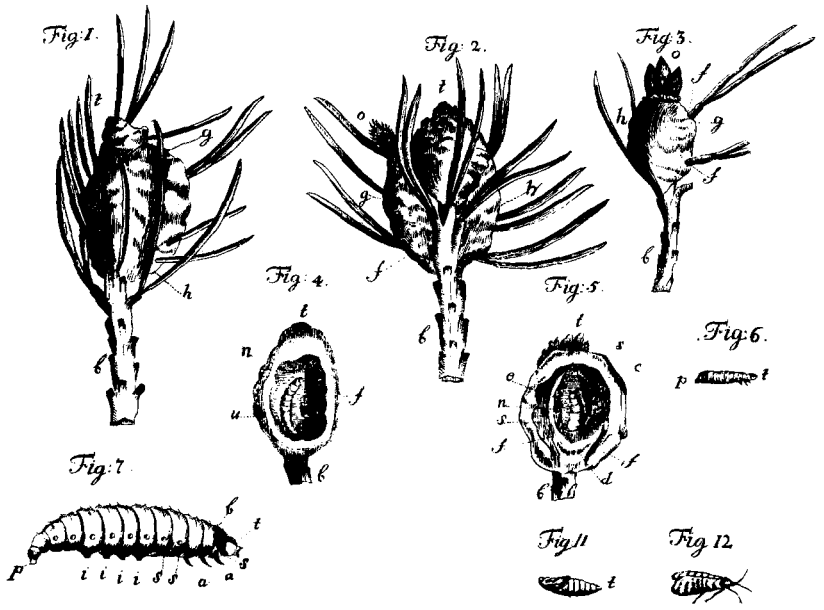


FIG. 1. Some of the illustrations provided by De Geer with his account of *Petrova resinella*. Intact resin galls are shown (1–3), as well as opened galls with the larva inside (4, 5), and drawings of the larva (6, 7), pupa (11), and adult (12). From De Geer (1776, volume 1, plate 33).

awareness of the special chemical peculiarities of resin, and it is this awareness that prompts him to wonder about the insect's seeming ability to tolerate the substance. Conifer resin, he states, like the odorous turpentine oil derived from it, is well known to be ill-tolerated by insects ("bekanntermassen . . . schlechterdings nicht ertragen . . ."). [2] How, then, can *Petrova* withstand living so closely exposed to resin ("mitten in einer harzigen Materie")?

As a start, he sought reassurance that the gall was constituted of resin. He found that it was soluble in alcohol ("Weingeist"), smelled of turpentine oil, and emitted upon ignition the odor of burned mastic ("Mastix"), a well-known resin product, commonly used as a varnish ingredient.

Then he experimented. He took a *Petrova* larva and placed it on a piece of paper soaked in turpentine oil. The larva crawled about and became visibly wetted with oil, but remained alive. A control arctiid caterpillar ("Bärraupe"), similarly placed, promptly became restless ("unruhig") and had to be herded

[2] Turpentine oil must have been in general use at the time. It was, for instance, recommended for entomological purposes by Hoppe (1752), who suggests brushing pinned beetles with the fluid to aid in their preservation.



FIG. 2. Typical resin gall of *Petrova resinella*. The gall on right is opened to show the larva within. Photos taken by author in summer near Saarwiesen, Germany.

by hand to be kept on the paper. It quickly weakened and within 4 min "gave up the ghost" ("gab . . . den Geist auf").

A further three *Petrova* larvae, placed in a closed jar with pieces of turpentine-soaked paper, fared well initially, but drowned overnight in excess oil. Proper precautions to prevent drowning showed yet another larva to emerge alive from two days of such confinement. Prolonged tolerance also was shown by a larva that was placed in a jar while still within the hollow of its partly opened gall, and therefore exposed to the vapors of the oil only. Two "smooth green caterpillars" confined as controls died in 2 minutes; a fly lasted only half an hour.

De Geer concludes succinctly that neither turpentine vapors nor turpentine oil ("weder Terpentinölgeruch, noch das öl") are fatal to *Petrova*. The larvae, he reasons, must be structured differently from other insects ("anders . . . eingerichtet"), in some fashion that he presumes to involve the respiratory system. He expresses regret that the larva's respiratory orifices and tubes ("Luftlöcher und Lungenröhren") are too small for him to examine in detail. He does not elaborate beyond this point, nor does he look for parallels with other insects. However, he obviously does raise the notion of food specialization and of adap-

tive tolerance by a herbivore of the noxious chemistry of its host. I know of no other comparably dated writings that address these issues. De Geer is remembered mostly as a prolific describer of insects. He was evidently otherwise insightful as well.

Acknowledgments—I am indebted to John G. Franclemont for helping to establish the identity of the larva.

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SAMPLING RANGE OF MALE SWEETPOTATO WEEVILS (*Cylas formicarius elegantulus*) (SUMMERS) (COLEOPTERA: CURCULIONIDAE) TO PHEROMONE TRAPS: INFLUENCE OF PHEROMONE DOSAGE AND LURE AGE

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Abstract—Studies were conducted to determine the effects of sex pheromone dosage and lure age on movement of male sweetpotato weevils (SPW), *Cylas formicarius elegantulus* (Summers), using mark–release–recapture techniques. SPW trap counts from various downwind distances were compared for dosages ranging from 0.01 to 10.0 μg and lure ages ranging from fresh (0 days old) to 64 days old. The percentages of male SPW recaptured decreased with an increase in release distance and decreased with a decrease in dosage at each corresponding distance. Most SPW were caught within the first 16-hr period. Slopes of percent recapture vs. release distance for the two higher dosages (10 μg and 1.0 μg) differed from those of the two lower dosages (0.1 and 0.01 μg) but did not differ from each other. Intercepts were similar among the three higher dosages. Slopes did not differ among the five lure ages examined. Intercepts differed between fresh (0 days old) and 24-day-old septa at 16 hr and between fresh (0 days old) and 34-day-old septa at 40 hr. Previous exposure to pheromone (conditioning) did not increase percentages of SPW recaptured. Results indicate that male SPW are capable of traversing distances of at least 280 m in 16 hr. The pheromone tested in this study appears to be effective at dosages lower than any other coleopteran sex-pheromone system. Incorporation of this pheromone into a SPW management system may effectively reduce the use of insecticides.

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Key Words—*Cylas formicarius elegantulus* (Summers), Coleoptera, Curculionidae, sex pheromone, sweetpotato weevil, mark–release–recapture.

INTRODUCTION

Mechanisms and rates of dispersal for *Cylas formicarius elegantulus* (Summers), the sweetpotato weevil (SPW), have been mostly speculative. Initially, SPWs were thought to fly only the short distances from infested sweet potato plantings to noninfested neighboring plants (Cockerham et al., 1954). Early mark–release–recapture studies in Louisiana indicated that SPW may be able to move distances of up to 2 km. However, since SPW released on individual dates were not marked differently, the time required to traverse this distance could not be determined (Deen, 1940; Cockerham et al., 1954).

Coffelt et al. (1978) recognized that a female SPW produced a sex pheromone that attracted only males. In subsequent laboratory studies, the active component of the pheromone was isolated, identified, synthesized, and bioassayed (Heath et al., 1986). Field trials in the U.S. Virgin Islands and Florida indicated that trap counts were positively correlated with pheromone dosage and that males could travel 70 m in 24 hr from the nearest source of weevils to a pheromone trap baited with 10 μg of synthetic sex pheromone (Proshold et al., 1986; Jansson et al., 1990). These studies indicated that this pheromone was attractive at low dosages. However, the range of attraction and sampling range for the pheromone was not determined.

Wall and Perry (1987) suggested that it is only through carefully timed mark–release–recapture experiments that reasonable evidence concerning the range of pheromone attraction can be acquired. The objective of this study was to examine the movement of male SPW to pheromone traps baited with different dosages of synthetic pheromone and lure ages by utilizing timed mark–release–recapture techniques. Through these studies we hoped to determine the sampling range of the pheromone for SPW.

METHODS AND MATERIALS

SPW males that had been trapped in commercial sweet potato [*Ipomoea batatas* (L.) Lam.] fields were held in the laboratory for one week. Males were then painted on the thorax with different colors of enamel paint, and released at various distances downwind from the pheromone source. The trap used in all experiments was a plastic funnel trap (Jansson et al., 1989). This trap was shown to be the most effective trap for male SPW in southern Florida (Jansson et al., 1989; Jansson and Heath, 1990; Jansson et al., unpublished data). Synthetic sex-pheromone [(*Z*)-3-dodecen-1-ol-(*E*)-2-butenoate) (99.9% pure) that

had been synthesized at the U.S.D.A., A.R.S., Insect Attractants, Behavior and Basic Biology Research Laboratory, Gainesville, Florida, was applied to methylene chloride-extracted rubber septa (Thomas Scientific, Swedesboro, New Jersey) as previously described (Heath et al., 1986).

Experiments were conducted during the months of July–October in fallow tomato, bean, and squash fields with no prior history of sweet potato planting. These fields were chosen because of their isolation from sweet potato fields that might harbor pheromone-producing, wild SPW females, lack of turbulence-producing obstructions, and lack of interference from nonmarked wild SPW males. Nighttime minimum temperatures ranged from 20°C to 24°C, daytime maximum temperatures ranged from 28°C to 33°C; winds were generally from the east–southeast.

Previous Exposure. To determine if prior exposure to pheromone may have conditioned males to the synthetic pheromone, laboratory-reared males (ages ranging from one to three weeks) with no prior exposure to synthetic pheromone were compared to pheromone-trap-collected males from commercial fields. One hundred males of each type were released simultaneously at distances of 25, 50, 75, and 100 m downwind from a pheromone source late in the afternoon (1630 hr EST). Three plastic funnel traps baited with 10 μg of synthetic pheromone were placed 12.5 m apart in a line perpendicular to the line of weevil release and the prevailing wind direction. Percentages recaptured were determined after 16 and 40 hr. The experiment was replicated five times.

Pheromone Dosage. The influence of pheromone dosage on the percentage of marked males recaptured was determined in the field. Rubber septa loaded with either 0.01, 0.1, 1.0, or 10.0 μg of synthetic pheromone were examined. Three plastic funnel traps baited with the same dosage of pheromone were used per release night. The three pheromone-baited traps were placed 12.5 m apart in a line perpendicular to the line of weevil release and the prevailing wind direction. Marked males (200 males/release distance) were released late in the afternoon (1630 hr EST) downwind from the pheromone traps. Release distances used were 3, 5, 10, 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 100, 120, 160, 200, 240, and 280 m, with up to seven release distances examined per night. The number of distances examined each night was limited by the number of paint colors available and the number of male weevils available for release. Each distance was tested at least twice. The number of males recaptured in the three traps were totaled 16 hr after male release. The males recaptured between 16 and 40 hr were recorded and added to the 16-hr total. Thus the 40-hr catch represents a cumulative total.

Lure Age. To determine if lure age influenced male response to pheromone, rubber septa loaded with 10 μg of synthetic sex pheromone were aged outdoors for either 0, 14, 24, 34 or 64 days. Each lure age was replicated four times. Experimental design was similar to that previously described, except that

only 100 male SPW were released at each distance and the distances tested were 25, 50, 75, and 100 m.

Data were analyzed by regressing arcsin transformed percentages of weevils recaptured on release distance for each male type (conditioned or nonconditioned), pheromone dosage, and lure age. Parameter estimates were compared between male types, among dosages and among lure ages within time periods, and between time periods within dosages and lure ages using the general linear test (Neter and Wasserman, 1974).

RESULTS

Previous Exposure. Laboratory-reared and pheromone-trap-collected males did not differ in their percent recapture to synthetic sex pheromone when released from various distances downwind of traps (16-hr slopes: $P = 0.4346$, $F_{1,52} = 0.62$; intercepts: $P = 0.6322$, $F_{1,52} = 0.23$; 40-hr slopes: $P = 0.4551$, $F_{1,52} = 0.57$; intercepts: $P = 0.6542$, $F_{1,52} = 0.20$). Thus, prior experience with the synthetic pheromone did not influence the percentage of male SPW recaptured.

Pheromone Dosage. Pheromone dosage significantly influenced percentage recapture of marked male SPW released at different distances downwind from the pheromone source. Percentages of SPW males recaptured decreased with an increase in release distance and decreased with a decrease in dosage at each corresponding distance (Figure 1). At distances of 280 m, percentage recaptured averaged ca. 10% at a dosage of 10 μg. For the 0.01-, 0.1-, and 1.0-μg dosages a 10% level of recapture was found at ca. 5, 70, and 120 m, respectively. The regression parameter estimates are given in Table 1. In general, slopes did not differ between 10- and 1-μg dosages at 16 and 40 hr, but the slopes of these two dosages differed from those of 0.10 and 0.01 μg at 16 hr and from that of 0.01 μg at 40 hr. Intercepts did not differ ($P > 0.05$) among the three higher dosages (10.0, 1.0, and 0.10 μg), but intercepts for these three dosages were significantly greater ($P < 0.05$) than that for the lowest dosage (0.01 μg) at both 16 and 40 hr. Release rate of the pheromone from rubber septa increases with an increase in dosage (Heath et al., 1990). Thus, it is doubtful that similar recapture rates for these three dosages (10.0, 1.0, and 0.10 μg) are due to similar release rates in the field. It is more likely that behavioral saturation to the pheromone is occurring. Most SPW were caught within the first 16 hr. This is similar to observations by Proshold et al. (1986), who found more SPW males were caught during the first night that pheromone traps were placed in sweet potato fields on the U.S. Virgin Islands. Parameter estimates were similar ($P > 0.05$) between the 16- and 40-hr catch for most dosages (Table 1). Only the intercept of the lowest dosage differed ($P < 0.05$) between time periods.

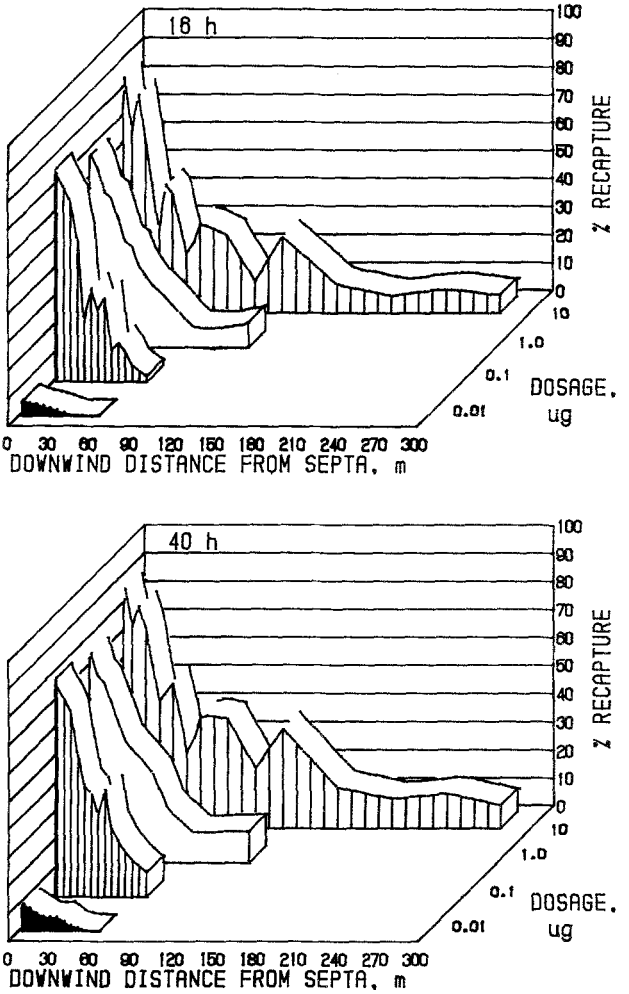


FIG. 1. Percentages of marked SPW males recaptured at 16 and 40 hr in plastic funnel traps baited with different dosages of synthetic sex pheromone when released at different distances downwind of the pheromone source.

These results indicate that male SPW are capable of traversing distances of up to 280 m in less than 16 hr. This is probably accomplished by very short trivial flights (Mason et al., unpublished data). When males were placed at each release site, they typically climbed to the top of the nearest vegetation and flew toward the trap within minutes after their release. The distances of these flights were generally 1–2 m.

TABLE 1. REGRESSION PARAMETER ESTIMATES FOR INFLUENCE OF SPW RELEASE DISTANCE ON PERCENTAGES RECAPTURED FOR EACH OF FOUR PHEROMONE DOSAGES AND TWO SAMPLING PERIODS

Dosage (μg)	Intercept (SE)	Slope (SE)	F	df	P	r^2
16 hr						
0.01	0.09 (0.007)a,A[a]	−0.051 (0.005)a,A	76.70	1,14	0.0001	0.84
0.10	1.35 (0.107)b,A	−0.702 (0.076)b,A	85.39	1,24	0.0001	0.78
1.00	1.13 (0.064)b,A	−0.512 (0.042)c,A	146.71	1,24	0.0001	0.85
10.00	1.27 (0.079)b,A	−0.518 (0.045)c,A	129.25	1,32	0.0001	0.80
40 hr						
0.01	0.14 (0.015)a,B	−0.073 (0.011)a,A	38.24	1,14	0.0001	0.73
0.10	1.32 (0.111)b,A	−0.629 (0.082)b,A	58.88	1,20	0.0001	0.74
1.00	1.23 (0.066)b,A	−0.540 (0.044)b,A	150.22	1,20	0.0001	0.86
10.00	1.35 (0.070)b,A	−0.522 (0.040)b,A	167.36	1,20	0.0001	0.83

[a] Parameter estimates followed by different lowercase letters within the same sampling period indicate significant differences among dosages, and estimates followed by different uppercase letters within the same dosage indicate significant differences between sampling periods [$P < 0.05$ general linear test (Neter and Wasserman, 1974)].

Lure Age. Percentages of marked SPW males recaptured were negatively correlated with release distance for each lure age. There were no significant differences between the slopes for the five lure ages examined according to a test for homogeneity of slopes ($P > 0.05$) (Table 2 and Figure 2). However, intercepts did differ among ages (Table 2). Intercepts differed between fresh lures (0 days old) and 24- and 64-day-old lures at 16 hr, but not at 40 hr. Intercepts for fresh lures did not differ from those of 14- and 34-day-old lures at 16 hr, and from those of 14-, 24-, and 64-day-old lures at 40 hr. The few differences found probably were due, in part, to environmental conditions. Lower percentages of males were recaptured during cooler and/or rainy weather. This indicates that environmental factors such as nighttime temperature and precipitation can influence male movement to pheromone traps (Mason et al. unpublished data). Currently, we are determining the influence of environmental factors such as temperature, precipitation, wind speed and direction, and biological factors, such as male age and reproductive status, on male movement to pheromone-baited traps. These data show that the attractiveness of synthetic pheromone formulated on methylene-chloride-extracted rubber septa to male SPW did not decrease for lure ages between 0 and 64 days old. Jansson et al. (1990) found that attractiveness of pheromone formulated on rubber septa did not decrease appreciably for lures between 0 and 34 days old.

TABLE 2. REGRESSION PARAMETER ESTIMATES FOR INFLUENCE OF SPW RELEASE DISTANCE ON PERCENTAGES RECAPTURED FOR EACH OF FIVE LURE AGES AND TWO SAMPLING PERIODS

Lure age (days)	Intercept (SE)	Slope (SE)	F	df	P	r^2
16 hr						
0	1.07 (0.16)a[a]	−0.47 (0.09)a	22.48	1,22	0.0001	0.51
14	1.28 (0.20)ab	−0.62 (0.12)a	24.41	1,22	0.0001	0.53
24	0.85 (0.11)b	−0.43 (0.07)a	43.33	1,22	0.0001	0.66
34	1.94 (0.09)ab	−0.47 (0.06)a	59.55	1,22	0.0001	0.73
64	0.87 (0.12)b	−0.45 (0.07)a	36.66	1,22	0.0001	0.63
40 hr						
0	0.98 (0.20)a	−0.40 (0.12)a	10.54	1,22	0.0001	0.32
14	1.39 (0.21)ab	−0.64 (0.13)a	23.53	1,22	0.0001	0.52
24	0.96 (0.13)ab	−0.47 (0.08)a	36.80	1,22	0.0001	0.63
34	0.65 (0.17)b	−0.30 (0.11)a	8.33	1,22	0.0001	0.28
64	1.15 (0.11)ab	−0.57 (0.07)a	63.67	1,22	0.0001	0.74

[a] Parameter estimates followed by different lowercase letters within the same sampling period indicate significant differences among dosages [$P < 0.05$ general linear test (Neter and Wasserman, 1974)].

DISCUSSION

Mark–release–recapture studies on long-range movement of insects to attractant sources are scarce. Wall and Perry (1987), in summarzing the literature for lepidopteran insects, found that many studies claiming to demonstrate the range of attraction (maximum distance over which insects show directed movement to a source) actually demonstrated sampling range (maximum distance in which insects are shown to reach a source in a given period of time). Unfortunately, the time required for insects to traverse this distance, even in sampling-range studies, is often neglected or broadly defined. Mark–release–recapture studies that are carefully timed, give the form and concentration of the attractant used, habitat information, and environmental conditions under which the experiment was conducted (all of which can influence pheromone dispersal and insect movement) are the most informative (Wall and Perry, 1987). While most work has concentrated on lepidopterans, especially noctuids, few studies have been published on coleopterans. Work on coleopterans has focused on bark beetles and the boll weevil (*Anthonomus grandis* Boheman), probably due to the devastating damage done by these insects, the ability of chemists to isolate an attractant and characterize it, and entomologists' ability to document

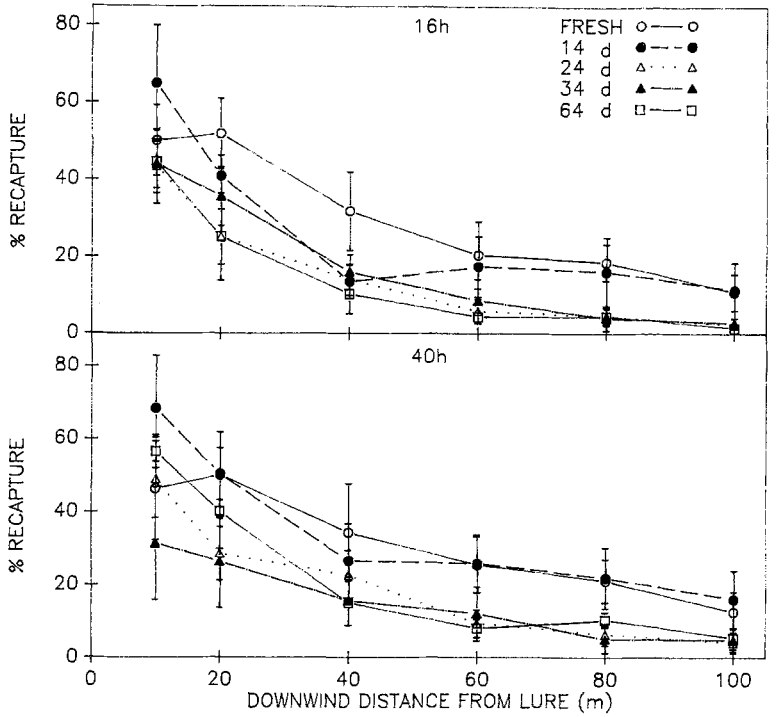


FIG. 2. Percentages of marked SPW males recaptured in plastic funnel traps baited with 10-μg lures of different ages when released at different distances downwind.

its efficacy in the field. In the current study we were able to determine that the sampling range for SPW is at least 280 m when a pheromone source of 10 μg is used as an attractant. Current behavioral work is underway to determine the absolute range of attraction for SPW.

Boll weevils are able to disperse great distances. The longest record is 72 km traversed by two weevils. These marked weevils were recovered in grandlure-baited traps 15–16 days (19.4 m/hr) and 29–33 days (9.6 m/hr) later (Johnson et al., 1976). This equaled a record for unmarked boll weevils in Mexico (Davich et al., 1970). Over a shorter time period, a marked male boll weevil traveled 400 m in 2–5 days (8.3–3.3 m/hr), while a marked female traveled 1600 m in 1–4 days (66–16 m/hr). Another female was able to traverse 272 m in 2 days (5.6 m/hr). In comparison, male SPW are able to traverse 280 m in under 16 hr (17.5 m/hr). This is comparable to the fastest rate found for the boll weevil (Johnson et al., 1976). Lepidoptera have very quick mark–release–recapture times (Baker and Roelofs, 1981; Karandinos, 1974; Kehat et al., 1976; Kishaba et al., 1970). Thirty percent of marked cabbage looper, *Trichoplusia*

ni (Hubner), males released 105 m downwind from a 25-mg pheromone source were recovered within 15 min (i.e., 420 m/hr) (Kishaba et al., 1970). At distances of up to 300 m, 6% were recovered in 15 min (1200 m/hr). Lesser peachtree borer males, *Synanthedon pictipes* (Grote and Robinson), have recapture rates of up to 66 m/hr when responding to traps baited with virgin females (Karandinos, 1974).

In laboratory bioassays of various insects, pheromone dosage is typically positively correlated with male response up to some threshold level (Coffelt and Burkholder, 1972; Shorey and Gaston, 1964). Similar trends have been found in the field. The percentages of males recovered in pheromone traps decreases as the downwind release distance increases and dosage decreases (Karandinos, 1974; Proshold et al., 1986). Although we found no differences between the slopes or intercepts for dosages within the range of 1.0–10.0 μg, there was a positive correlation between dosage and percentage recapture of SPW males within the range of 0.01–1.0 μg. The dosages that we tested are considerably lower than those dosages used in other coleopteran trapping studies. For instance, 0.4–25 mg of grandlure was used in boll weevil dose–response tests (Hardee et al., 1974). Schlyter et al. (1987) used dosages in the range of 0.5–500 mg (release rate/day) for 2-methyl-3-buten-2-ol and 0.01–10 mg (release rate/day) for *cis*-verbenol to examine attraction of *Ips typographus* (L.). Thus, compared to other coleopteran systems, SPW males are extremely sensitive to the female pheromone.

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INTERFERENCE IN DETERMINATION OF RUBIDIUM AND CESIUM IN FISH AND ZOOPLANKTON BY GRAPHITE FURNACE ATOMIC ABSORPTION SPECTROMETRY

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Abstract—Interference in the determination of rubidium and cesium in fish and zooplankton was examined by graphite furnace atomic absorption spectrometry using synthetic matrices of these organisms at known concentrations and dilutions. Absorbance signals were depressed at dilution factors of less than 100, which confirms that the method is not free of interference for determination of rubidium and cesium in fish and zooplankton.

Key Words—Rubidium determination, cesium determination, atomic absorption spectrometry, fish, zooplankton, chemical interference.

INTRODUCTION

In contrast to flame atomic absorption spectrometry (AAS), chemical interference appears to be minimal when measuring rubidium in biological materials using graphite furnace AAS (Ramirez-Munoz, 1968; Price, 1972; Tanaka et al., 1977; Grobenski et al., 1983). In addition, ionization of rubidium and cesium in the graphite furnace has been found to be insignificant at 2300°C (Sturgeon and Berman, 1981). However, in the case of an inorganic substance, signal depression of cesium because of matrix effects has been observed in a U.S. Geological Survey (USGS) rock sample when using graphite furnace AAS (Grobenski et al., 1983). These authors resolved the problem by adding sulfuric acid to the rock sample solutions.

Measurement of stable rubidium and cesium in fish and their food has been

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suggested as a method of determining whether their diet consists mainly of zooplankton or benthic prey (Kanevskii and Fleishman, 1972). Measurement of stable cesium in fish also has been undertaken to better understand the behaviour of ^{137}Cs (Kolehmainen, 1972; Hakonson et al., 1971). However, chemical interference when measuring stable rubidium and cesium in fish by graphite furnace has received little attention.

Chiasson (1986) found that in juvenile sockeye salmon (*Oncorhynchus nerka*) and threespine stickleback (*Gasterosteus aculeatus*) rubidium and cesium absorbance signals were suppressed at low sample dilutions when using graphite furnace AAS. This paper quantifies this phenomenon by examining the absorbance signals of rubidium and cesium in synthetic fish and zooplankton matrices of known composition and dilutions using graphite furnace AAS. Potential solutions to the problem are discussed.

METHODS AND MATERIALS

Apparatus. Rubidium and cesium were measured with a Perkin-Elmer (P-E) 603 flameless atomic absorption spectrophotometer equipped with a HGA-2200 graphite furnace. Electrodeless discharge lamps (P-E) were powered by an electrodeless discharge lamp high voltage supply and shaded by a red filter. Pyrolytically coated graphite tubes were used for all determinations. Atomization temperatures were calibrated with a silicon dioxide photodiode temperature sensor. Peak heights were transcribed by a 056 P-E recorder with a PRS printer sequencer with 10-sec integration.

Optimum Operating Conditions. Operating conditions are presented in Table 1. Samples were run at least three times or until a deviation of 10% or less of the mean was obtained. To ensure that memory effect was not present, blanks were included after every 10 samples in all runs. To reduce errors, pipetted volumes were never less than 10 μl and volumes in flasks were never less than 50 ml.

Reagents. Solutions containing rubidium and cesium were prepared from BDH Suprapur RbCl and Analar reagent CsCl. All other reagents were of special grades. The HNO_3 concentration in standards was matched to the concentrations in the samples.

Preparation of Biological Samples. Juvenile sockeye salmon, threespine stickleback, and zooplankton were collected from Kennedy Lake, Vancouver Island, Canada (latitude 49°22′N, longitude 125°15′W). Zooplankton and eviscerated fish were dried to constant weight at 80°C and ground separately in a stainless-steel mill. Samples of 200 mg were then placed in 20-ml scintillation vials to which were added 1 ml of HNO_3 and 9 ml distilled deionized water. The solutions were evaporated to dryness over a hot plate, redissolved in 1 ml

TABLE 1. OPTIMAL SETTINGS FOR MEASUREMENT OF RUBIDIUM AND CESIUM

Parameter	Rb	Cs
Wavelength (nm)	780.0	852.1
Slit (nm)	4.0	4.0
EDL (watts, power)	2.0	3.9
Drying time (sec)	40	40
Drying temperature (°C)	110	110
Drying ramp (sec)	22	2.2
Charring time (sec)	40	40
Charring temperature (°C)	1000	1000
Charring ramp (sec)	32	32
Atomization time (sec)	7	7
Atomization temperature (°C)	2400	2400
Argon gas flow	80[a]	80[b]
Sensitivity[c]	0.0010	0.0020

[a] Argon gas flow 7 sec normal during atomization.
[b] Argon gas flow 3 sec interrupt during first stages of atomization. Rate of flow was optimized.
[c] Sensitivity in μg/ml for peak absorbance of 0.00044 with 20 μl injection with a Perkin-Elmer autosampler.

of HNO_3, sealed, and allowed to stand overnight. The vials were then opened, the solutions brought quickly to a boil, allowed to cool, then diluted to 5 ml with distilled deionized water.

Effect of Dilution on Absorbance Signal of Rubidium. Individual 200-mg samples of sockeye, stickleback, and zooplankton were digested in HNO_3 and subsequently diluted to 5 ml with distilled deionized water to yield a dilution factor of 25. Aliquots were extracted and diluted to 75 and 125 times the initial sample concentration for fish and to 50, 75, and 150 the initial sample concentration for zooplankton. The absorbance signal of each solution was measured, multiplied by the dilution factor, and plotted against the sample dilution (Figure 1). Absorbance signal increased with increasing sample dilution. In the absence of experimental error and interference, absorbance signal strength should have remained constant within each sample type. This confirmed that signal strengths were suppressed at lower dilution factors for both fish and zooplankton.

Effect of Sodium Chloride on Signal Strength. Sodium and chloride are probably major ions in both threespine stickleback and juvenile sockeye (Copeland and Ayers, 1972; Copeland et al., 1973). To determine if the addition of NaCl to samples would depress the absorption signals of rubidium and cesium, two calibration standards of 25, 50, and 100 ng/ml of each element were dissolved in deionized water. To one series of calibration standards for each element, 1000 μg/ml of NaCl was added. The remaining two received no NaCl

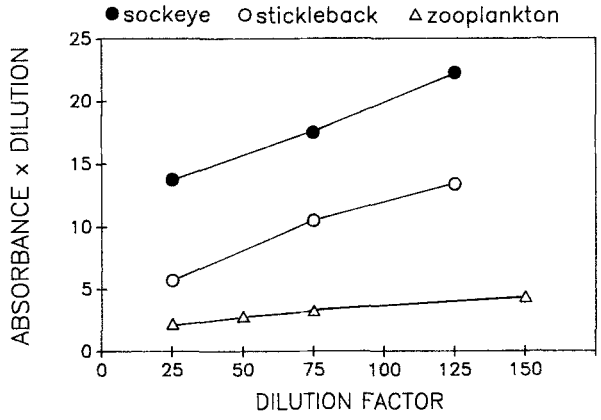


FIG. 1. Absorbance versus dilution for analysis of rubidium in sockeye, stickleback, and zooplankton.

and were used for comparison. The addition of NaCl resulted in a depression of absorbance signal strength (Figure 2).

Dilution Factor and Matrix Effects. Rather than attempt the identification of all elements that contributed to the chemical interference at low dilution factors and their possible removal from the matrix, a closer examination of the effect of dilution factor on absorption signal strength was undertaken. Synthetic fish and zooplankton matrices were prepared based on the elements present at concentrations greater than 10 μg/g wet weight in zooplankton and coho salmon (*Oncorhynchus kisutch*) from Lake Michigan (Copeland and Ayers, 1972; Copeland et al., 1973) (Tables 2 and 3). Salts were dissolved in a solution of

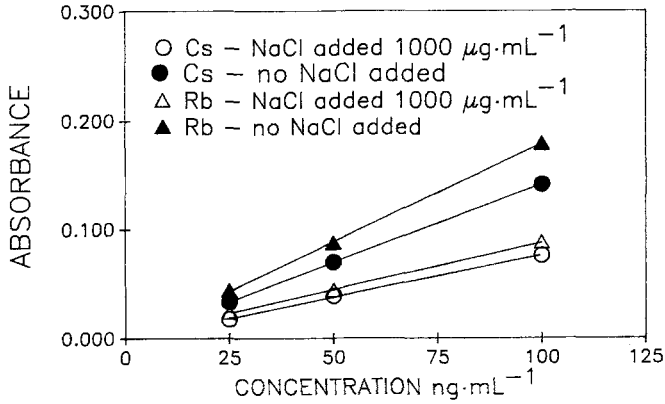


FIG. 2. Absorbance for rubidium and cesium alone and spiked with 100 μg/ml NaCl.

TABLE 2. ARTIFICIAL ZOOPLANKTON MATRIX

Major element (μg/g wet weight)		Source	Weight used (g)[a]
Al	99	Al_2O	1.87
Br	88	KBr	1.31
Ca	1750	$CaCl_2 2H_2O$	9.74
		$CaCO_3$	25.88
K	970	KBr	as above[b]
		KNO_3	24.04
Cl	1440	NaCl	6.76
		$MgCl_2 6H_2O$	20.06
		$CaCl_2 2H_2O$	as above
Na	265	NaCl	as above
Mg	240	$MgCl_2 6H_2$	as above

[a] Final concentrations are adjusted to dry weight using an 80% loss in weight of sample. Only elements present in concentrations greater than 10 μg/g were used. Final volume was 2 liters.
[b] Part of the element is derived from a compound(s) listed above.

1800 ml of distilled deionized water and 200 ml of HNO_3 to match acid concentrations in actual fish and zooplankton samples. Synthetic matrices were chosen over actual biological samples to enable manipulation of individual elements in future experiments. Separate aliquots of the synthetic fish matrix were diluted with distilled deionized water to obtain four solutions with the following dilution factors: 10, 50, 100, and 200. Rubidium and cesium then were added to the solutions to obtain concentrations of 10, 25, 50, and 100 ng/ml for each dilution factor. Therefore, four separate concentrations of rubidium and cesium

TABLE 3. ARTIFICIAL FISH MATRIX

Major elements (μg/g wet weight)		Source	Weight used (g)[a]
Ca	238	$CaCl_2$	6.48
Cl	707	NaCl	3.53
		$CaCl_2$	as above[b]
K	3588	KNO_3	9.27
Na	501	NaCl	as above
		$NaHCO_3$	9.91
Mg	216	MgO	3.58

[a] Final concentrations are adjusted to dry weight using an 80% loss in weight of sample. Only elements present in concentrations greater than 10 μg/g wet weight were used. Final volume was 2 liters.
[b] Part of the element is derived from a compound(s) listed above.

were prepared for each dilution factor. The same procedure was followed for the synthetic zooplankton matrices. To determine absorbance signal strength in the absence of matrix effects, four standards consisting of 10, 25, 50, and 100 ng/ml of rubidium and cesium were prepared in distlled deionized water.

Interference was evident in fish and zooplankton matrices at dilution factors of less than 100 for both rubidium and cesium (Figure 3). The degree of chemical interference can be assessed by examining absorbance values for the standards with those for the various dilution factors. Data were fitted to a regression equation derived by Chiasson (1986) and coefficients were computed using a routine by Becker and Chambers (1984), which minimizes the difference between the sum of squares of the deviations of the observed and predicted values. This was done for both rubidium and cesium and for each matrix type. The equation is of the form:

$$z = \frac{y}{A(0) + \frac{A(1)}{x} + y\left[B(0) + \frac{B(1)}{x}\right]}$$

where z is absorbance, y is dilution, x is concentration, and $A(0)$, $A(1)$, $B(0)$, and $B(1)$ are coefficients. The coeffcients giving the best fits are listed in Table 4. The coefficients of determination for each regression were highly signficant.

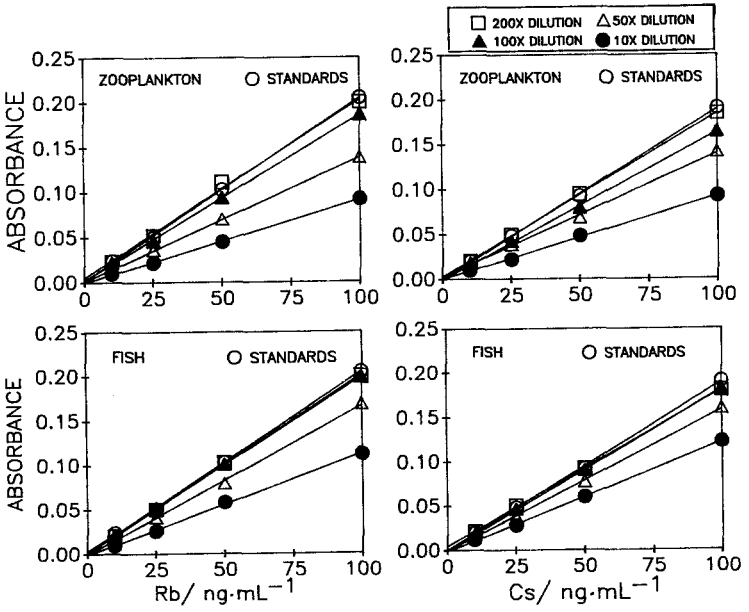


FIG. 3. Absorbance versus concentration of rubidium and cesium at various dilutions for synthetic fish and zooplankton matrices.

TABLE 4. COEFFICIENTS OF REGRESSION

	Zooplankton		Fish	
	Rubidium	Cesium	Rubidium	Cesium
A(0)	19	15	−0.30	−66
A(1)	5600	4900	5800	3500
B(0)	0.42	0.82	0.98	0.99
B(1)	440	470	490	450
r^{2a}	0.96	0.92	0.75	0.92
F^b	336.0	161.0	42.0	161.0
df	1,14	1,14	1,14	1,14
P	<0.01	<0.01	<0.01	<0.01

[a] r^2 = coefficients of determination.
[b] F = F test, all highly significant, values followed by degrees of liberty and probabilities.

DISCUSSION

A suppression of absorbance signal strength at lower dilution factors in synthetic fish and zooplankton matrices verifies that determination of rubidium and cesium in biological materials of this type cannot be regarded as free of chemical interference for routine analysis. The presence of NaCl in natural fish and zooplankton is a factor in this phenomenon as its addition to solutions of rubidium and cesium results in a suppression of signal strength.

Chemical interference when using electrothermal atomizers has been noted for a number of biological substances (Delves, 1982). In such cases, suppression of signal strength may be attributable to the inorganic constituents present during atomization (Delves, 1982). Matrix matching and matrix modification have been suggested as methods leading to improved analysis when using flameless AAS (Farley and Nelson, 1982). This approach could be applied to analysis of rubidium and cesium in fish and zooplankton by graphite furnace AAS. However, the determination of an appropriate matrix adds an additional burden and source of error. In instances where rubidium and cesium are in sufficiently high concentration, increased dilution of the sample to 100 would appear to minimize the problem. Concentration of rubidium and cesium are detectable in juvenile sockeye and threespine stickleback at dilutions of 100. However, in cases where initial concentrations of rubidium and cesium are low, increased dilution of samples may not be acceptable because of reduced signal strength. Investigation of potential chemical interference in measurement of rubidium and cesium in biological material by graphite furnace AAS is warranted before proceeding with analysis of previously unexamined cases.

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ANTIFEEDANT ACTIVITY AGAINST *Spodoptera litura* LARVAE AND [^{13}C]NMR SPECTRAL ASSIGNMENTS OF THE MELIATOXINS

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Abstract—The tetranortriterpene, meliatoxin A_2, induced a significant level of antifeedant activity in *Spodoptera litura F.* larvae, while meliatoxin B_1, which lacks the ring D epoxide, was much less active. Complete [^{13}C]NMR assignments for both compounds derived from homo- and heteronuclear short- and long-range couplings are given.

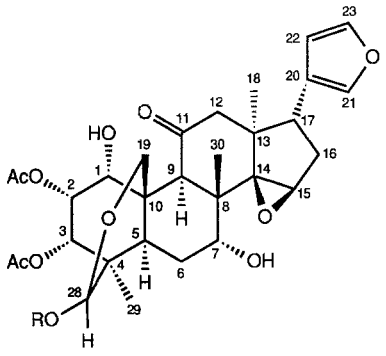
Key Words—Meliatoxins, *Melia azedarach*, antifeedant activity, [^{13}C]NMR spectroscopy, *Spodoptera litura.*

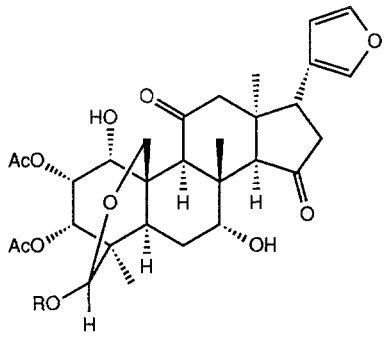
INTRODUCTION

It is well known that certain Meliaceae, Cneoraceae, and Rutaceae species are not affected by insects because of the presence of tetranortriterpenes (limonoids), which possess insect antifeedant activity (Connolly, 1983; Dreyer, 1983; Taylor, 1983). Recent investigations of these naturally occurring compounds have been directed towards their potential use as pest-control chemicals (Champagne et al., 1989). Our investigations into the toxic constituents of the fruit of *Melia azedarach* L. var. *australasia* (Meliaceae), revealed that four closely related tetranortriterpenes, meliatoxins A_1 **(1)**, A_2 **(2)**, B_1 **(3)**, and B_2 **(4)** were responsible for many of the symptoms resulting from ingestion of whole fruit by animals (Oelrichs et al., 1983).

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1 R = $CH_3CH_2CH(CH_3)CO$ (4' 3' 2' 1')

2 R = $(CH_3)_2CHCO$ (2' 1')

3 R = $CH_3CH_2CH(CH_3)CO$-

4 R = $(CH_3)_2CHCO$-

SCHEME 1.

Since these compounds are limonoids and bear a close structural resemblance to the trichilins, some of which are known to possess insect antifeedant activity (Nakatani et al., 1981), it was considered worthwhile to investigate and compare the antifeedant activity of two of the meliatoxins, A_2 and B_1, which were available in the largest quantity. In addition, we have extended our earlier [^{1}H]NMR studies of these compounds to include complete ^{13}C assignments for meliatoxins A_2 and B_1.

METHODS AND MATERIALS

Test Material. Meliatoxin A_2 (**2**) and meliatoxin B_1 (**3**) isolated from the plant *Melia azedarach* L. var. *australasia* (Oelrichs et al., 1983) were used for the study. [^{1}H] and [^{13}C]NMR spectra were recorded on a Varian model VXR 300S spectrometer. Unambiguous ^{1}H and ^{13}C chemical shifts were obtained using various two-dimensional NMR techniques such as the ^{1}H–^{1}H Cosy experiment (both short- and long-range variations), as well as ^{13}C–^{1}H correlation spectra (Hetcor) and its long-range analog. The long-range Cosy experiment was optimized for $^4J_{H\text{-}H}$ = 1–3 Hz. The long-range Hetcor was optimized for $^2J_{C\text{-}H}$ = 9 Hz.

Rearrangement of meliatoxin A_1 to B_1 was achieved when freshly distilled boron trifluoride etherate (0.02 ml) was added to a stirred solution of meliatoxin A_1 (3.66 mg) in dry toluene (0.5 ml) at 0°C under argon. After 5 min, dry pyridine (0.16 ml) was added and the volatile solvents evaporated under high vacuum. Toluene (0.5 ml) was added and evaporated once more. The residue

was filtered through a short column of silica (1 : 1 ethyl acetate–dichloromethane), and the eluate was evaporated. Reversed-phase chromatography (μBondapak, 3 : 2 acetonitrile–water) gave pure meliatoxin B_1 (2.1 mg), identical with an authentic sample by TLC, [^{1}H]NMR, MS, and CD.

Insect Rearing. Second-instar *Spodoptera litura*, F. larvae of 9–11 mg body weight were used throughout the study; these were reared and maintained at 27 $\pm$ 1°C, 70–75% relative humidity, and 16 hr photophase. Three replicates of 10 larvae per treatment were employed for each concentration used.

Bioassay. Compounds were emulsified with 10 μg Noigen FNV emulsifier (Dai Ichi Co., Bombay, India) and 100, 200, 300, 400, and 500 ppm dilutions in water were prepared just prior to use. Leaf disks of 25 mm^2 in size were punched out from castor leaves (*Ricinus communis*), and the candidate compounds (approx. 0.3 ml/disk) were applied as previously described (Koul, 1987). Control disks were treated with water plus emulsifier alone. The leaf disks were allowed to dry in the shade at room temperature and then placed in large (20-cm-diameter) Petri dishes on a wet filter paper disk of the same size to prevent desiccation of leaf disks. The experiments were conducted as choice and no-choice tests, the former with an equal number of treated and untreated disks per arena (Koul, 1987). However, the total number of disks in both experimental layouts was constant. After each 24-hr period, the disks were removed and replaced with similarly treated and untreated disks. The leaf area consumed in each treatment was measured with a photometric device (Koul, 1987) after each 24-hr period up to 72 hr and consumption was calculated as percentage of controls in both tests.

In addition, the larvae in each experiment were weighed every 24 hr in order to tabulate the weight gain. Data were analyzed by analysis of variance.

RESULTS AND DISCUSSION

[^{13}C]NMR Assignments. Table 1 lists the ^{13}C chemical shifts and their assignments for meliatoxins A_1, A_2, B_1, and B_2. Proton chemical shifts and their assignments have been published previously (Oelrichs et al., 1983). The information derived from the ^{1}H–^{1}H Cosy and ^{13}C–^{1}H Hetcor experiments allowed unambiguous chemical shift assignments for all protons and carbons except for the methyl groups and the quaternary carbons. To distinguish these signals, long-range Cosy and Hetcor experiments were conducted on meliatoxins A_2 and B_1, and the following information was obtained. Long-range coupling in the modified Cosy experiment ($^4J_{H\text{-}H}$) showed correlations from the proton at H-28 to the methyl protons at 0.84 ppm corresponding to the ^{13}C chemical shift at 18.6 ppm. Similar couplings were observed from the protons at H-12 to the methyl singlet at 1.24 ppm corresponding to the ^{13}C chemical

TABLE 1. [^{13}C]NMR ASSIGNMENTS FOR MELIATOXINS **1** TO **4**

Carbon	**1**	**2**	**3**	**4**
C-1	71.77d	71.79d	71.91d	71.90d
C-2	68.54d	68.58d	68.77d	68.78d
C-3	73.26d	73.21d	73.35d	73.28d
C-4	40.9s	40.9s	40.7s	40.7s
C-5	28.03d	28.06d	28.08d	28.08d
C-6	25.97t	25.92t	23.01t	22.98t
C-7	70.87d	70.85d	69.26d	69.23d
C-8	42.3	42.1s	43.16s	43.16s
C-9	48.37d	48.36d	51.36d	51.35d
C-10	42.0s	41.9s	42.66s	42.65s
C-11	211.14s	211.12s	209.11s	210.00s
C-12	48.54t	48.54t	43.32t	43.32t
C-13	43.6s	43.5s	44.9s	44.8s
C-14	72.60s	72.62s	62.38d	62.38d
C-15	57.82d	57.82d	219.15s	219.53s
C-16	32.11t	32.11t	50.50t	50.47t
C-17	39.17d	39.17d	41.28d	41.78d
C-18	20.96q	20.95q	28.08q	28.08q
C-19	64.43t	64.46t	63.85t	63.86t
C-20	123.13s	123.12s	121.77s	121.76s
C-21	139.7d	139.5d	140.3d	140.2d
C-22	110.70d	110.70d	110.26d	110.25d
C-23	143.3d	143.0d	143.4d	143.4d
C-28	93.56d	93.58d	93.07d	93.08d
C-29	18.88q	18.79q	18.46q	18.43q
C-30	21.72q	21.75q	20.18q	20.18q
$\underline{C}H_3CO$-2	20.71q	20.72q	20.77q	20.77q
$CH_3\underline{C}O$-2	168.77s	168.84s	169.00s	169.05s
$\underline{C}H_3CO$-3	20.71q	20.72q	20.92q	20.92q
$CH_3\underline{C}O$-3	169.98s	170.00s	169.96s	169.96s
C-1′	175.36s	175.77s	175.37s	175.77s
C-2′	41.04d	34.15d	41.02d	34.10d
C-3′	26.60t		26.54t	
CH_3-2′	16.30q	18.74q	16.32q	18.74q
		18.74q		18.66q
C-4′	11.34q		11.34q	

shift at 21.0 ppm. H-30 (1.09 ppm) must by default correspond to C-30 at 21.6 ppm.

The long-range version of the Hetcor demonstrated coupling from the methyl protons on C-18 (21.0 ppm) to the quaternary carbon at 43.5 ppm, thus establishing it as C-13. The protons corresponding to C-29 (18.6 ppm) were

coupled to the quaternary carbon at 40.9 ppm, establishing C-4. The protons of C-30 (21.6 ppm) were coupled to the quaternary carbon at 42.1 ppm establishing C-8. By default, C-10 must then correspond to the remaining quaternary carbon at 41.9 ppm. This last chemical shift was further verified by its long-range $^2J_{C\text{-}H}$ coupling to the H-19 protons.

Antifeedant Testing. Meliatoxin A_2 reduced the ingestion of food by *Spodoptera litura* larvae significantly ($P < 0.05$) after 24 hr in a no-choice test at 400 and 500 ppm concentrations (Figure 1). This compound also exhibited severe feeding deterrence at 300–500 ppm in choice tests (Figure 2), completely reducing the consumption. However, at 300 ppm the older larvae (72 hr) could feed on the treated disks at the same rate as those of the control disks. Similarly, meliatoxin B_1 reduced feeding by nearly one half of the controls, showing a feeding range of 50–60% of controls in a choice test after 24 hr. However, in a no-choice situation, consumption over a 72-hr period was 50–85.7% of controls (Figure 2). Although feeding was not reduced to the extent observed with meliatoxin A_2 treatment, the decrease in weight gain was highly significant ($P < 0.05$), particularly at the 400- and 500-ppm treatments (Figures 3 and 4). This shows that meliatoxin B_1 has a growth inhibitory effect rather than a clear antifeedant activity. The growth also was reduced significantly in *S. litura* larvae under the influence of meliatoxin A_2, but in this case it is due apparently to the decrease in consumption rate. At the 400- and 500-ppm levels, the average weight gain in A_2-treated larvae was significantly reduced ($P < 0.05$), reaching a low of 62.3 and 33.8 in the no-choice tests and 80.8 and 79.3 mg

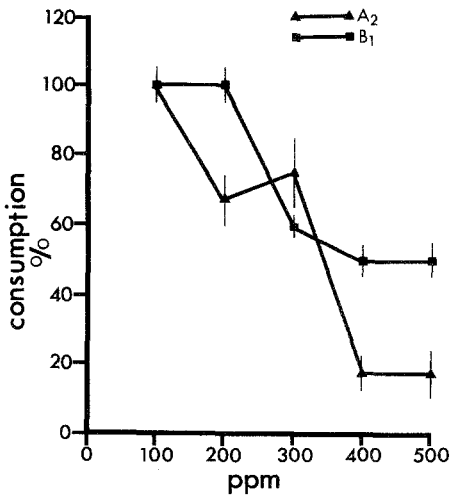


FIG. 1. Consumption (percent of controls) at various concentrations of meliatoxin A_2 and meliatoxin B_1 after 24 hr of treatment in a no-choice test.

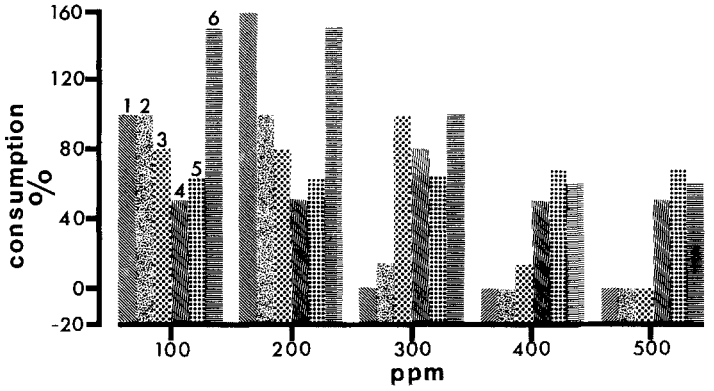


FIG. 2. Consumption (percent of controls) at various concentrations of meliatoxin A_2 (1–3) and meliatoxin B_1 (4–6) after 24 hr (columns 1 and 4), 48 hr (columns 2 and 5), and 72 hr (columns 3 and 6), respectively, in a choice test.

in the choice tests respectively, against 145.7 and 134.4 mg in controls after 72 hours (Figure 3 and 4).

These data clearly indicate that meliatoxin A_2 induces an antifeedant effect in *S. litura* larvae in both choice and no-choice tests with a significant threshold activity at 300 ppm. This is in accordance with the activity of closely related

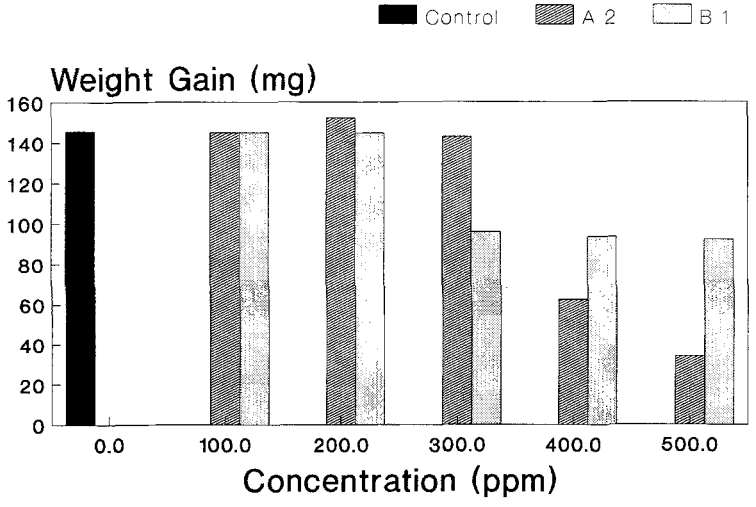


FIG. 3. Average weight gain of *Spodoptera litura* larvae after 72 hr of treatment of meliatoxin A_2 and meliatoxin B_1 in a no-choice test.

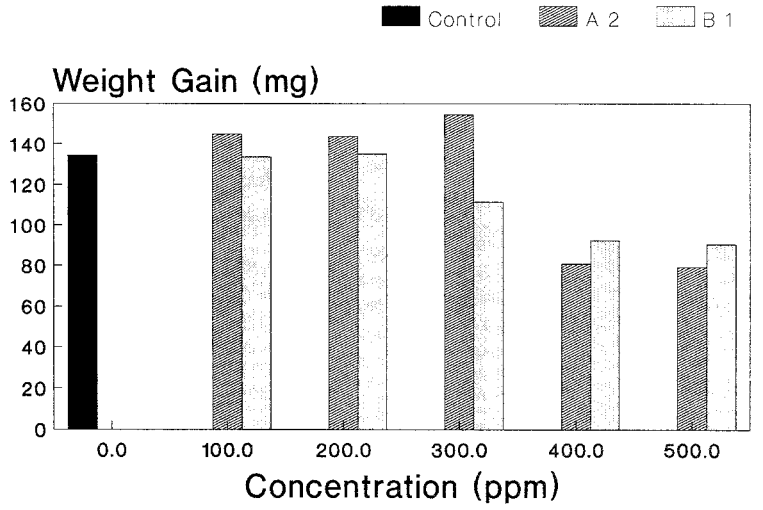


FIG. 4. Average weight gain of *Spodoptera litura* larvae after 72 hr of treatment of meliatoxin A_2 and meliatoxin B_1 in a choice test.

trichilin-D, which is known to induce antifeedance in *Spodoptera eridania* at 400 ppm (Nakatani et al., 1981). The two compounds both lack the 12-OH substituent, which is considered to be important for candidate activity (Nakatani et al., 1981). It may not, however, be the only functionality that induces feeding deterrence (Koul, 1983) but could be one of the necessary functions for maximum activity in this type of limonoid.

According to the present study, it appears that even the epoxide function on ring D has an important role to play, as is evident from the variable activities of meliatoxins A_2 and B_1. Meliatoxin B_1 has a keto function on ring D in place of the epoxide ring and is less active as an antifeedant than meliatoxin A_2. The epoxide and furan groups also have been shown as essential structural requirements for high antifeedant activity in citrus limonoids (Bentley et al., 1988) where deepoxidation of limonin, for instance, reduced the activity considerably. It is, however, probable that the keto function may be responsible for inducing growth inhibition rather than having an antifeedant effect. However, this remains speculation and needs further structure–activity relationship studies. When treated with BF_3-etherate, meliatoxin A_1 was converted stereospecifically to meliatoxin B_1 indicating that the latter compound, together with meliatoxin B_2, may be artifacts formed during the extraction and purification procedure.

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β-PHELLANDRENE: KAIROMONE FOR PINE ENGRAVER, *Ips pini* (Say) (COLEOPTERA: SCOLYTIDAE)

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Abstract—The responses of *Ips pini* (Scolytidae) to multiple-funnel traps baited with the pheromone, ipsdienol, and various monoterpenes were determined in stands of lodgepole pine in southern and central British Columbia. *Ips pini* was attracted to both ipsdienol and β-phellandrene, demonstrating that β-phellandrene is a kairomone for this species. *Lasconotus complex* (Colydiidae) and a *Corticeus* sp. (Tenebrionidae) were attracted to both ipsdienol and β-phellandrene. The *Corticeus* sp. exhibited a synergistic response to the combination of ipsdienol and β-phellandrene; the responses of the other two species to the combination were additive. The predators, *Thanasimus undatulus*, *Enoclerus sphegeus*, and *E. lecontei* (Cleridae), were attracted to ipsdienol-baited traps, while *Monochamus clamator* (Cerambycidae) and *Dendroctonus ponderosae* (Scolytidae) were attracted to β-phellandrene. Attraction of all eight species increased with increasing release rates of ipsdienol and/or β-phellandrene.

Key Words—Kairomone, β-phellandrene, ipsdienol, monoterpenes, *Ips pini*, Coleoptera, Scolytidae, predators, competition, primary attraction, dose response.

INTRODUCTION

The mass aggregation phenomenon, typical of many subcortical feeding insects, has long been known for the pine engraver, *Ips pini* (Say). Anderson (1948) found that *I. pini* were attracted, in large numbers, to bolts of jackpine, *Pinus banksiana* Lambert, infested with *I. pini*. The principal semiochemical involved in mass aggregation was identified as the male-produced pheromone, ipsdienol

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(2-methyl-6-methylene-2,7-octadien-4-ol) (Vité et al., 1972; Stewart, 1975; Birch et al., 1980; Lanier et al., 1980).

Although evidence of the use of pheromones by *I. pini* has been documented, very little has been gleaned on the use of host-produced kairomones in facilitating mass aggregation. Monoterpenes are abundant in the phloem tissue of lodgepole pine, *P. contorta* var. *latifolia* Engelmann (Mirov, 1961; Shrimpton, 1972, 1973), a common host for *I. pini* (Furniss and Carolin, 1980), and are used as attractive kairomones by several species of Scolytidae (Borden, 1982; Dickens et al., 1984; Byers et al., 1985, 1988; Schroeder and Eidmann, 1987; Schroeder, 1988; Volz, 1988; Chénier and Philogène, 1989; Schroeder and Lindelöw, 1989; Miller and Borden, 1990). β-Phellandrene is the major constituent monoterpene in the phloem tissue of lodgepole pine (Shrimpton, 1972). Four species of bark beetles that typically breed in lodgepole pine are attracted to β-phellandrene (Miller and Borden, 1990). We hypothesized, therefore, that β-phellandrene should act as a kairomone for *I. pini*, either as an attractant or repellent, and either alone or in synergy with the pheromone, ipsdienol.

METHODS AND MATERIALS

Chemicals. (+)-3-Carene, (+)-limonene, (−)-β-phellandrene, racemic α-pinene, (−)-β-pinene, and terpinolene (chemical purities, all >95%) were obtained from H.D. Pierce, Jr. (Department of Chemistry, Simon Fraser University). The chiralities of β-phellandrene and β-pinene are predominantly (−) in lodgepole pine (Mirov, 1961). β-Myrcene and racemic ipsdienol (chemical purities, both 98%) were obtained from Phero Tech Inc. (Vancouver, British Columbia).

Release Devices. In experiment 1, monoterpenes were released from closed, polyethylene microcentrifuge tubes (400 μl) (Evergreen Scientific, Los Angeles, California), each filled with a single monoterpene. The release rates for α-pinene, β-pinene, myrcene, 3-carene, limonene, β-phellandrene, and terpinolene were approximately 8.9, 9.3, 22.3, 22.9, 25.5, 29.3, and 29.5 mg/day, respectively, at 27°C (determined by weight loss). In experiment 2, β-phellandrene was released in two fashions to obtain two different release rates: (1) two closed, polyethylene microcentrifuge tubes (each 400 μl) per trap; and (2) one closed, polyethylene screw-cap bottle (15 ml) (Ampak Inc., Richmond, British Columbia) per trap. The release rates were approximately 59 and 450 mg/day, respectively, at 27°C. In experiment 3, β-phellandrene was released in three fashions: (1) one open, polypropylene microcentrifuge tube (1.5 ml) (Quality Scientfic Plastics, Petaluma, California) per trap, containing five 2-cm-long glass capillaries (ID 1.5 mm; OD 1.8 mm), each sealed at one end

and filled with β-phellandrene; (2) five closed, polyethylene microcentrifuge tubes (1.8 ml) (Evergreen Scientific) per trap; and (3) one closed, polyethylene screw-cap bottle (15 ml) per trap. The release rates were approximately 3, 40, and 450 mg/day, respectively, at 27°C.

Ipsdienol was released from 10-cm-lengths of C-flex® tubing (ID 1.6 mm; OD 3.2 mm) (Concept Inc., Clearwater, Florida), filled with a solution of ipsdienol in ethanol (chemical purity, 99%), and pressure-sealed at both ends. In experiments 1–2, the release rate of ipsdienol was approximately 0.6 mg/day at 24°C. The release rates for the devices used in experiment 3 (approximately 6, 60, and 600 μg/day at 24°C) were obtained by varying the concentration of ipsdienol in ethanol.

Trapping Experiments. In all experiments, grids of multiple-funnel traps (Lindgren, 1983) were set in mature stands of lodgepole pine. Replicate grids were placed at least 100 m apart, and traps were spaced 10–15 m apart within each replicate. Each trap was suspended between trees by rope such that the top funnel of each trap was 1.3–1.5 m above ground. No trap was within 2 m of any tree. Sexes of captured *I. pini* were determined using declivital characters (Lanier and Cameron, 1969).

The effects of various monoterpenes were tested in experiment 1 in order to distinguish the effect of β-phellandrene relative to other monoterpenes common in lodgepole pine (Shrimpton, 1972). Five replicates of eight 8-unit traps per replicate, were set near Princeton, British Columbia, in grids of 2 × 4, from May 29 to July 2, 1987. The treatments were as follows: (1) ipsdienol alone, (2) ipsdienol with α-pinene, (3) ipsdienol with β-pinene, (4) ipsdienol with 3-carene, (5) ipsdienol with myrcene, (6) ipsdienol with terpinolene, (7) ipsdienol with β-phellandrene, and (8) ipsdienol with limonene.

Experiment 2 tested for effects of β-phellandrene as a kairomone in primary and secondary attraction of *I. pini*, as well as for interactions between ipsdienol, β-phellandrene and locality. Three replicates of six 12-unit traps per replicate were set in grids of 2 × 3 at each of three localities in British Columbia in 1988: (1) near Princeton from August 24 to September 4, (2) near Jaffray from August 25 to September 27, and (3) near Williams Lake from August 27 to 31. The treatments were as follows: (1) blank control, (2) β-phellandrene (59 mg/day), (3) β-phellandrene (450 mg/day), (4) ipsdienol alone, (5) ipsdienol with β-phellandrene (59 mg/day), and (6) ipsdienol with β-phellandrene (450 mg/day).

Experiment 3 tested for response of *I. pini* to different doses of ipsdienol and β-phellandrene, as well as for interaction between ipsdienol, β-phellandrene, and locality. Two replicates of nine 12-unit traps per replicate were set in grids of 3 × 3 at each of three localities in British Columbia in 1988: (1) near Princeton from August 19 to September 1, (2) near Jaffray from August 25 to September 27, and (3) near Williams Lake from August 27 to 31. The

treatments were the nine binary combinations of ipsdienol (6, 60, and 600 μg/day) with β-phellandrene (3, 40, and 450 mg/day), with each of the release rates for a given chemical occurring only once in any row or column of each grid.

Statistical Analyses. The data were analyzed using the SAS statistical package version 5.0 (SAS Institute Inc., Cary, North Carolina). When necessary, trap catch data were transformed to remove heteroscedasticity. In experiment 1, trap catches of *I. pini* were transformed by $\sqrt[3]{Y}$. In experiment 2, catches of all species were transformed by $\ln(Y + 1)$. In experiment 3, catches of (1) *Thanasimus undatulus* Say (Cleridae) and *Lasconotus complex* LeConte (Colydiidae) were transformed by $\sqrt[3]{Y}$, (2) *Dendroctonus ponderosae* Hopkins (Scolytidae) were transformed by $-1/\sqrt{Y}$, and (3) *I. pini* and a *Corticeus* sp. (Tenebrionidae) were transformed by $\ln(Y + 1)$. Sex ratio data for *I. pini* were transformed by $\arcsin\sqrt{Y}$. Homoscedastic data were subjected to either one-way, two-way, or three-way full-factorial ANOVA. Block effects were not considered in the analyses. Three orthogonal contrasts were performed for experiment 1 while Duncan's multiple-range tests were used in experiments 2 and 3 when $P < 0.05$.

RESULTS

β-Phellandrene had a significant effect on trap catches of *I. pini* in all three experiments. In experiment 1, catches to traps baited with ipsdienol and β-phellandrene were significantly higher than catches to traps baited with ipsdienol and any other monoterpene (Figure 1). There were no significant differences between the ipsdienol treatment and either the ipsdienol + β-phellandrene treatment (orthogonal contrast, $F(1, 32)$, $P = 0.226$) or the remaining treatments (orthogonal contrast, $F(1, 32)$, $P = 0.488$).

Jaffray data were omitted from analyses in experiments 2 and 3 because only 12 *I. pini* were caught. In experiment 2, β-phellandrene alone significantly increased trap catches (Table 1, Figure 2A,C). In Princeton, traps with baits releasing β-phellandrene at a high rate were preferred over those with a low release rate, while catches in blank control traps were intermediate (Figure 2A). In Williams Lake, traps with baits releasing β-phellandrene at either rate were preferred over blank controls (Figure 2C). There was an additive, not synergistic, interaction between ipsdienol and β-phellandrene on the response of *I. pini* (Table 1). Significant interactions occurred between location and β-phellandrene treatments, and between location, β-phellandrene, and ipsdienol treatments (Table 1).

In experiment 3, responses of *I. pini* increased as the release rates of ips-

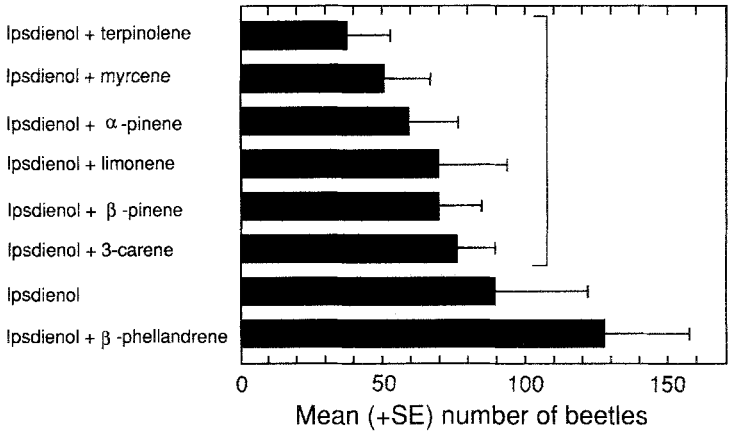


FIG. 1. The effect of various monoterpenes on the attraction of *Ips pini* to ipsdienol-baited multiple-funnel traps in experiment 1 near Princeton, BC, from May 24 to July 2, 1987. Means grouped by a line are significantly different from the treatment of ipsdienol + β-phellandrene at $P = 0.027$ [orthogonal contrast, ANOVA, $F(1, 32)$, on data transformed by $\sqrt[3]{Y}$ $(N = 5)$].

TABLE 1. ANALYSIS OF VARIANCE ON EFFECTS OF LOCATION (PRINCETON AND WILLIAMS LAKE, BC), IPSDIENOL (0.6 mg/day), AND β-PHELLANDRENE (59 and 450 mg/day) ON NUMBER AND SEX RATIO OF *Ips pini* RESPONDING TO MULTIPLE-FUNNEL TRAPS (EXPERIMENT 2, 1988)

	Trap catch[a]			Proportion of males[b]		
Source	*df*	*F*	*P*	*df*	*F*	*P*
Location (A)	1	32.34	<0.001	1	0.49	0.493
Ipsdienol (B)	1	281.46	<0.001	1	12.17	0.003
β-Phellandrene (C)	2	5.66	0.010	2	0.45	0.647
A * B	1	1.79	0.193	1	0.02	0.896
A * C	2	5.07	0.015	2	0.38	0.692
B * C	2	1.71	0.203	2	2.68	0.094
A * B * C	2	2.94	0.072	0		
Error	24			19		

[a] Data transformed by $\ln(Y + 1)$.
[b] Data transformed by $\arcsin\sqrt{Y}$.

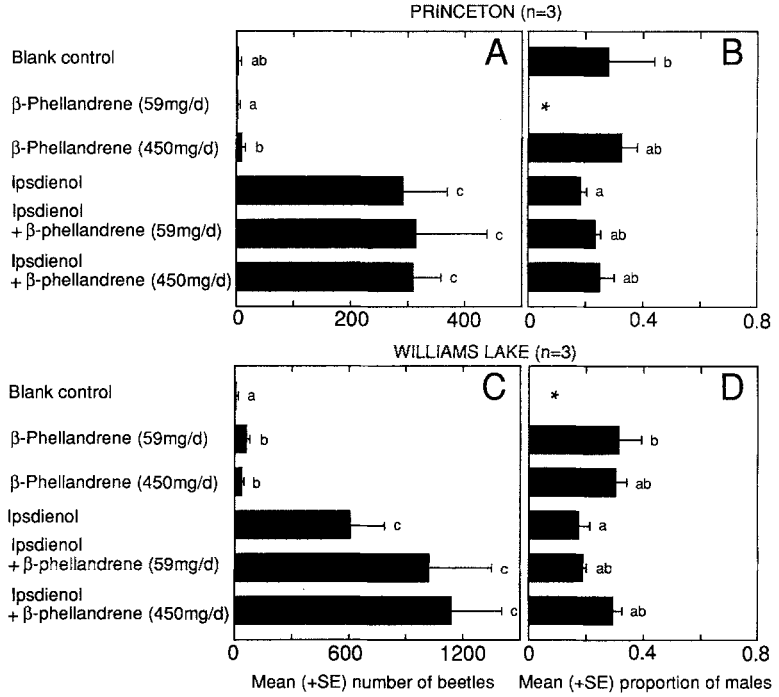


FIG. 2. The effect of β-phellandrene, with or without ipsdienol, on the attraction (A,C) and sex ratio (B,D) of *Ips pini* responding to multiple-funnel traps in experiment 2 near Princeton (A,B) and Williams Lake (C,D) from August 24 to September 4, 1988 and August 27 to 31, 1988, respectively. Mean trap catches from the same location followed by the same letter are not significantly different at $P = 0.05$ [Duncan's multiple-range test on data transformed by ln $(Y + 1)$]. Mean proportions of males, in traps at the same location, followed by the same letter are not significantly different at $P = 0.05$ (Duncan's multiple-range test on data transformed by arcsin$\sqrt{Y}$). The proportions of males for treatments with low trap catches (*) were not included in the analyses.

dienol and β-phellandrene increased (Table 2, Figure 3A,B). There were significant differences between Princeton and Williams Lake in the magnitude of the increase (Table 2). However, in both cases the preferred treatments were those with high release rates of ipsdienol and β-phellandrene (Duncan's multiple-range test, $P = 0.05$).

The effect of β-phellandrene on the sex ratio of responding *I. pini* was variable. In experiment 1, the proportion of males in trap catches (mean ± SE $= 0.38 \pm 0.013$) was not affected by the presence of monoterpenes [ANOVA, $F(7, 32)$, $P = 0.582$]. In experiment 2, the presence of ipsdienol in traps significantly reduced the proportion of males (Table 1, Figure 2B,D). β-Phellandrene did not affect the sex ratio of beetles caught, although the interaction

TABLE 2. ANALYSIS OF VARIANCE ON EFFECTS OF LOCATION (PRINCETON AND WILLIAMS LAKE, BC), RELEASE RATE OF IPSDIENOL (6, 60, and 600 μg/day), AND OF β-PHELLANDRENE (3, 40, and 450 mg/day) ON NUMBER AND SEX RATIO OF *Ips pini* RESPONDING TO MULTIPLE-FUNNEL TRAPS (EXPERIMENT 3, 1988)

	Trap catch[a]			Proportion of males[b]		
Source	*df*	*F*	*P*	*df*	*F*	*P*
Location (A)	1	15.45	0.001	1	5.18	0.035
Ipsdienol (B)	2	48.08	<0.001	2	17.79	<0.001
β-Phellandrene (C)	2	17.57	<0.001	2	0.64	0.538
A * B	2	4.93	0.020	2	0.16	0.857
A * C	2	0.83	0.454	2	0.46	0.641
B * C	4	0.73	0.582	4	1.32	0.299
A * B * C	4	0.56	0.698	4	0.96	0.456
Error	18			18		

[a] Data transformed by $\ln(Y + 1)$.
[b] Data transformed by $\arcsin\sqrt{Y}$.

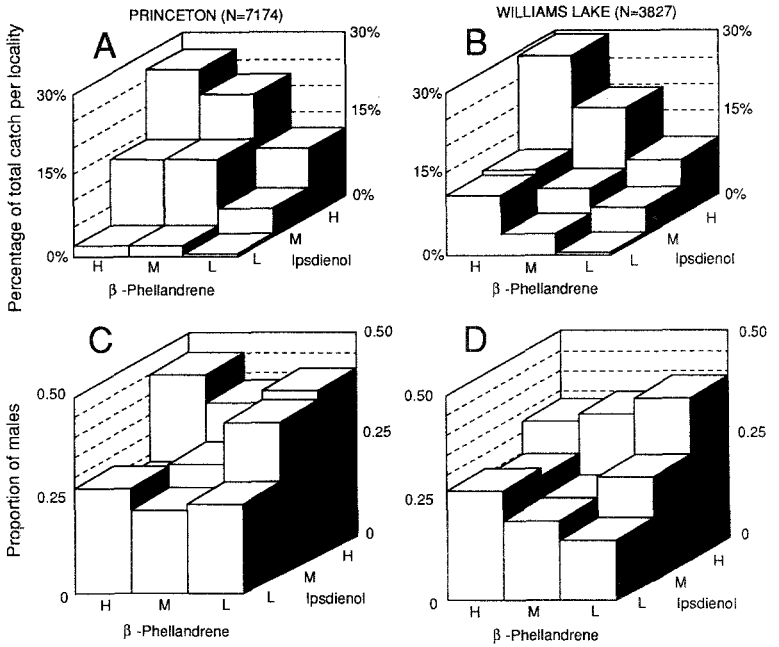


FIG. 3. The interaction between β-phellandrene and ipsdienol on the attraction (A,B) and sex rato (C,D) of *Ips pini* responding to multiple-funnel traps in experiment 3 near Princeton (A,C) and Williams Lake (B,D) from August 19 to September 1, 1988 and August 27–31, 1988, respectively. Release rates were 3(L), 40(M), and 450(H) mg/day for β-phellandrene and 6(L), 60(M), and 600(H) μg/day for ipsdienol.

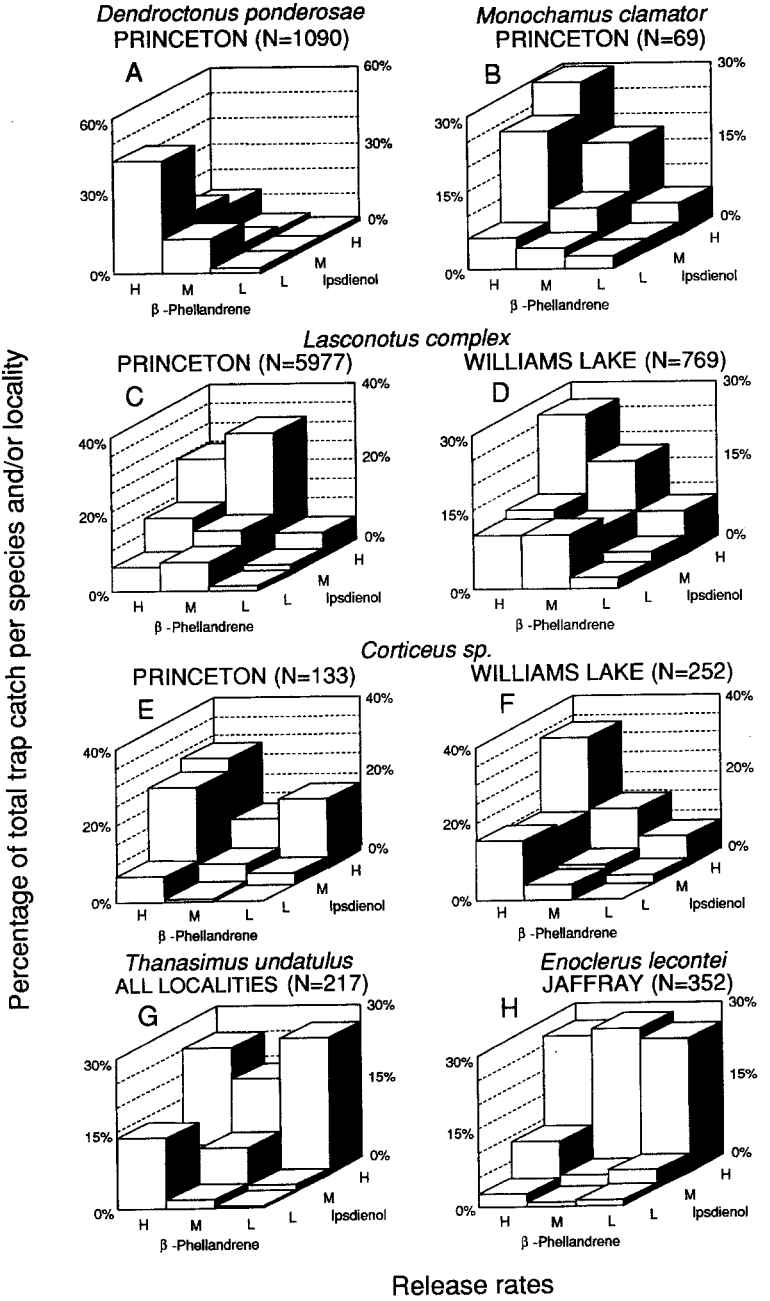

Dendroctonus ponderosae
PRINCETON (N=1090)
A
Monochamus clamator
PRINCETON (N=69)
B
Lasconotus complex
PRINCETON (N=5977)
C
WILLIAMS LAKE (N=769)
D
Corticeus sp.
PRINCETON (N=133)
E
WILLIAMS LAKE (N=252)
F
Thanasimus undatulus
ALL LOCALITIES (N=217)
G
Enoclerus lecontei
JAFFRAY (N=352)
H
β -Phellandrene
Ipsdienol
H
M
L
Percentage of total trap catch per species and/or locality
Release rates

TABLE 3. ANALYSIS OF VARIANCE ON EFFECTS OF RELEASE RATE OF IPSDIENOL (6, 60, and 600 μg/day) AND OF β-PHELLANDRENE (3, 40, and 450 mg/day) ON ATTRACTION OF *D. ponderosae*, *E. lecontei*, AND *M. clamator* TO MULTIPLE-FUNNEL TRAPS (EXPERIMENT 3, 1988)

	Dendroctonus ponderosae[a]			*Enoclerus lecontei*[b]			*Monochamus clamator*[a]		
Source	*df*	*F*	*P*	*df*	*F*	*P*	*df*	*F*	*P*
Ipsdienol (a)	2	1.92	0.208	2	129.43	<0.001	2	2.32	0.154
β-Phellandrene (B)	2	28.11	<0.001	2	1.28	0.325	2	3.28	0.085
A * B	4	0.23	0.912	4	0.95	0.478	4	0.58	0.683
Error	8			9			9		

[a] Princeton, BC.
[b] Jaffray, BC.

between ipsdienol and β-phellandrene was weakly significant. In contrast, the proportion of males caught in traps in experiment 3 increased with an increase in release rate of ipsdienol but showed no effect due to the release rate of β-phellandrene (Table 2, Figure 3C,D).

The mountain pine beetle, *Dendroctonus ponderosae*, was the only other scolytid trapped in large numbers (Figure 4A). In experiment 3, catches of *D. ponderosae* increased as the release rate of β-phellandrene increased (Table 3, Figure 4A). Conn et al. (1983) found that β-phellandrene had no effect on *D. ponderosae*. However, they employed low release rates (7 mg per day), comparable to our lowest release rate which showed little effect.

In experiments 2 and 3, species of Cleridae, Colydiidae, Tenebrionidae, and Cerambycidae (Coleoptera) showed significant responses to the presence of β-phellandrene and/or ipsdienol. Clerids showed a consistent preference for ipsdienol in both experiments. In experiment 2, *T. undatulus*, *Enoclerus sphegeus* F., and *E. lecontei* Wolcott were caught preferentially in traps baited with ipsdienol (Table 4). In experiment 3, *T. undatulus* and *E. lecontei* showed increased

FIG. 4. The interaction of β-phellandrene and ipsdienol on the attraction of *D. ponderosae* (Scolytidae), *M. clamator* (Cerambycidae), *L. complex* (Colydiidae), a *Corticeus* sp. (Tenebrionidae), *T. undatulus* (Cleridae), and *E. lecontei* (Cleridae) to multiple-funnel traps in experiment 3 near Princeton, Jaffray, and/or Williams Lake, BC, from August 19 to September 1, 1988, August 25 to September 27, 1988, and August 27 to 31, 1988, respectively. Release rates were 3(L), 40(M) and 450(H) mg/day for β-phellandrene and 6(L), 60(M), and 600(H) μg/day for ipsdienol.

TABLE 4. EFFECT OF β-PHELLANDRENE WITH AND WITHOUT IPSDIENOL ON IPSON ATTRACTION OF *L. complex*, *T. undatulus*, *E. sphegeus*, AND *E. lecontei* TO MULTIPLE-FUNNEL TRAPS (EXPERIMENT 2, 1988)

	Mean (± SE) catch per week[a]			
	Lasconotus complex[b]	*Thanasimus undatulus*[c]	*Enoclerus sphegeus*[d]	*Enoclerus lecontei*[e]
Blank control	0.4 ± 0.29 a	0.3 ± 0.20 a	0.0 ± 0.00 a	0.1 ± 0.08 a
β-Phellandrene (59 mg/day)	1.2 ± 1.17 a	0.1 ± 0.14 a	1.2 ± 0.58 ab	0.1 ± 0.08 a
β-Phellandrene (450 mg/day)	17.5 ± 7.99 b	0.7 ± 0.44 a	1.2 ± 0.58 ab	0.6 ± 0.46 ab
Ipsdienol	141.1 ± 69.80 c	3.7 ± 0.98 b	6.4 ± 3.09 c	2.8 ± 0.90 bc
Ipsdienol + β-phellandrene (59 mg/day)	350.9 ± 129.71 cd	1.8 ± 0.47 b	5.3 ± 2.02 bc	5.6 ± 2.51 c
Ipsdienol + β-phellandrene (450 mg/day)	377.5 ± 118.31 d	3.7 ± 1.49 b	9.3 ± 2.92 c	2.7 ± 1.34 abc

[a] Catches were standardized for an interval of one week. Means within a column followed by different letters are significantly different at $P = 0.05$ [Duncan's multiple-range test on data transformed by $\ln(Y + 1)$].
[b] Pooled for Princeton and Williams Lake, BC ($N = 6$).
[c] Pooled for Princeton, Jaffray, and Williams Lake, BC ($N = 9$).
[d] Williams Lake, BC ($N = 3$).
[e] Jaffray, BC ($N = 3$).

attraction to traps as the release rate of ipsdienol increased (Tables 3 and 5, Figure 4G,H). There were no significant effects due to locality, β-phellandrene, or interactions.

In contrast, two other potential predators or competitors, *L. complex* (Colydiidae) and a *Corticeus* sp., nr. *strublei* Blaisdell, (Tenebrionidae) were affected by both ipsdienol and β-phellandrene. In experiment 2, significantly more *L. complex* were caught in traps baited with ipsdienol than in traps not baited with ipsdienol (Table 4). More beetles were caught in traps with β-phellandrene released at a high rate than in the blank controls; the most preferred treatment was ipsdienol with a high release rate of β-phellandrene. The interaction between β-phellandrene and ipsdienol was additive, not synergistic or inhibitory [ANOVA, $F(2, 24)$, $P = 0.191$]. In experiment 3, trap catches of both *L. complex* and the *Corticeus* sp. increased as release rates of ipsdienol and β-phellandrene increased (Figure 4C–F). There were significant interactions between location and both ipsdienol and β-phellandrene for *L. complex* (Table 5). As in experiment 2, the interaction between ipsdienol and β-phellandrene was additive for *L. complex*. However, the interaction between ipsdienol and β-phellandrene had a synergistic effect on catches of the *Corticeus* sp. The combination resulted in trap catches greater than the sum of the proportional increases. The increased attraction of *Monochamus clamator* (LeConte) (Cerambycidae) to increasing release rates of β-phellandrene was weakly significant (Table 3, Figure 4B).

TABLE 5. ANALYSIS OF VARIANCE ON EFFECTS OF LOCATION, RELEASE RATE OF IPSDIENOL (6, 60, and 600 μg/day) AND OF β-PHELLANDRENE (3, 40, and 450 mg/day) ON ATTRACTION OF *T. undatulus*, *L. complex*, AND *Corticeus* SP. (TENEBRIONIDAE) TO MULTIPLE-FUNNEL TRAPS (Experiment 3, 1988)[a]

	Thanasimus undatulus[b]			*Lasconotus complex*[b]			*Corticeus* sp.[c]		
Source	*df*	*F*	*P*	*df*	*F*	*P*	*df*	*F*	*P*
Location (A)	2	2.87	0.074	1	88.44	<0.001	1	131.36	<0.001
Ipsdienol (B)	2	19.27	<0.001	2	18.45	<0.001	2	67.61	<0.001
β-Phellandrene (C)	2	1.08	0.353	2	21.37	<0.001	2	127.86	<0.001
A * B	4	1.37	0.270	2	4.63	0.024	2	5.28	0.23
A * C	4	0.60	0.663	2	5.45	0.014	2	3.54	0.62
B * C	4	0.82	0.525	4	0.16	0.955	4	11.01	<0.001
A * B * C	8	0.56	0.798	4	0.35	0.841	3	0.53	0.669
Error	27			18			12		

[a] Locations were Princeton, Jaffray, and Williams Lake, BC, for *T. undatulus*, and Princeton and Williams Lake, BC, for *L. complex* and *C. strublei*.
[b] Data transformed by $\sqrt[3]{Y}$.
[c] Data transformed by $\ln(Y + 1)$.

DISCUSSION

Our results support the hypothesis that β-phellandrene is used as a host kairomone by *I. pini*. Traps with baits releasing β-phellandrene at a high rate were more attractive to *I. pini* than blank control traps (Fig. 2A,B). β-Phellandrene significantly increased catches of *I. pini* to traps baited with ipsdienol (Table 1, Figures 2C, 3A,B) except for one location on one occasion when it failed to substantiate these results (Figure 2A). Conclusive support of this hypothesis requires the determination of the volatiles actually released from host material suitable for *I. pini*.

The effect of the interaction between ipsdienol and β-phellandrene on the response of *I. pini* was additive, according to ANOVA. Similarly, *I. latidens* showed an additive effect to the combination of its pheromone, ipsenol, and β-phellandrene (Miller and Borden, 1990). There is no evidence of either synergy or saturation in responses by either species. In both cases, the proportional increase in response due to the presence of β-phellandrene was the same with the respective pheromone as without it.

β-Phellandrene is a general kairomone for many species breeding in lodgepole pine. In addition to *I. pini*, we found that *D. ponderosae*, *L. complex*, a *Corticeus* sp., and *M. clamator* also were attracted to β-phellandrene. Miller and Borden (1990) found that *I. latidens*, *I. mexicanus* (Hopkins), *Hylastes longicollis Swaine*, and *Hylurgops porosus* (LeConte) also were attracted to traps baited with β-phellandrene. All nine species breed in lodgepole pine and are attracted by the most abundant monoterpene in its phloem tissue (Shrimpton, 1972). It seems probable that β-phellandrene also should be important for many other species breeding in conifers with high proportions of β-phellandrene in their phloem tissues. β-Phellandrene has the potential of becoming an important and critical component of many commercial baits for bark beetles, particularly since predators such as clerids are not attracted (Tables 3–5). Unfortunately, β-phellandrene is not commercially available.

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EFFECTS OF PROTEIN AND JUGLONE ON GYPSY MOTHS: GROWTH PERFORMANCE AND DETOXIFICATION ENZYME ACTIVITY

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Abstract—The individual and interactive effects of dietary protein and juglone on larval performance and midgut detoxification enxymes were investigated for the gypsy moth, *Lymantria dispar*. The experimental design was a 2 × 3 factorial, with two levels of protein and three levels of juglone. We monitored survival/development rates from egg hatch to pupation and conducted fourth-instar feeding trials for determination of nutritional indices. Enzyme solutions were prepared from midguts of fifth instars and assayed for polysubstrate monooxygenase, esterase, quinone reductase, and glutathione transferase activities. Results showed that low protein levels prolonged development times, increased consumption rates, and reduced pupal weights. Juglone markedly reduced survival, growth, and consumption rates, increased development times, and reduced pupal weights. The interaction between protein and juglone influenced larval digestion efficiencies and female pupal weights. Polysubstrate monooxygenase activities were unaffected by diet, whereas esterase activities increased in response to both low dietary protein and presence of juglone. Low protein levels increased soluble quinone reductase activities but decreased glutathione transferase activities. Glutathione transferase activities were lowest in larvae fed low-protein, high-juglone diets and may have contributed to the especially poor performance of larvae on those diets. Quinone reductase and glutathione transferase are the systems of importance in detoxification of juglone, and moderate to low activities of these enzymes may explain why gypsy moths perform poorly on members of the Juglandaceae.

Key Words—Detoxification enzymes, glutathione transferase, gypsy moth, Juglandaceae, juglone, *Lymantria dispar*, Lepidoptera, Lymantriidae, nutritional ecology, protein, quinone, quinone reductase.

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INTRODUCTION

The suitability of plants as food for phytophagous insects is determined by characteristics of both the plant and the herbivore. Nutrient, allelochemical, and structural components influence susceptibility of plants to herbivores. Biochemical, physiological, and behavioral adaptations influence the ability of insects to utilize particular plants as food. These characteristics vary both within and among plant and animal species and account for much of the diversity of plant–herbivore associations evident today.

In recent years, research on effects of diet quality on herbivore performance has begun to move from simple, single-factor studies to multiple-factor studies (e.g., Lincoln et al., 1982; Berenbaum and Neal, 1985; Broadway and Duffey, 1988; Johnson and Bentley, 1988). Although experimentally more complex, this approach is also more ecologically relevant in that it enables assessments of interactions among factors that affect herbivore performance. Indeed, such interactive effects have typically been observed, but the biochemical or physiological bases for the interactions are usually obscure.

One mechanism likely responsible for interactive effects between nutrients and allelochemicals is diet-induced changes in enzymatic detoxification systems. The influence of essential nutrients on the efficacy of insect detoxification enzymes is, surprisingly, virtually unknown. Numerous studies with mammals, however, have documented altered enzyme activities as a consequence of nutrient deficiencies. For example, inadequate dietary protein generally reduces cytochrome P-450 concentrations and monooxygenase activity (Dauterman, 1980; Boyd and Campbell, 1983). Such effects are likely to occur in insects as well. Given that enzymatic detoxification is a primary adaptation of insects to plant allelochemicals (Ahmad et al., 1986) and that many plant tissues provide food of marginal nutritional quality (Slansky and Scriber, 1985), diet-induced changes in enzyme function are likely to influence insect performance.

The research reported here was conducted to assess the direct and interactive effects of a nutrient (protein) and allelochemical (juglone) on detoxification enzyme activity and larval performance of the gypsy moth, *Lymantria dispar* (L.). Juglone, a 1,4-naphthoquinone, is a common constituent of members of the Juglandaceae, occurring freely as well as in glycosylated and reduced (1,4,5-trihydroxynaphthalene) forms (Gilbert et al., 1967; Hedin et al., 1979, 1980). We selected this system because: (1) dietary protein is known to influence gypsy moth performance (Rossiter, 1987); (2) juglone is a major secondary metabolite in species of *Carya* and *Juglans* that are moderately acceptable or avoided by gypsy moths (Lechowicz and Maufette, 1986), indicating that juglone may be toxic to the insects; and (3) enzymatic pathways most likely involved in detoxification of juglone are known (Wadleigh and Yu, 1987; Yu, 1987). We predicted that juglone would be toxic to gypsy moths, as evidenced

by reduced survival, growth, and pupal weights, and that consumption of low-protein diets would augment the toxicity of juglone as a result of decreased activity of detoxification enzymes. We were particularly interested in dietary effects on quinone (carbonyl) reductase and glutathione transferase activities, as these enzymes are the ones most likely to be involved in metabolism of juglone in gypsy moths.

METHODS AND MATERIALS

Insects. Gypsy moth egg masses were obtained from the Beneficial Insects Research Laboratories (USDA), Newark, Delaware. Larvae were reared in Percival growth chambers in the gypsy moth quarantine facility of the University of Wisconsin, Madison. Larvae were maintained at 25°C on a 16:8 hr light–dark cycle.

Artificial Diets. Diets were adapted from the high wheat germ formulation of ODell et al. (1985). Methyl paraben, a diet preservative and potential inducer of detoxification enzymes, was not used. Diets were autoclaved to inhibit growth of mold. We used the standard formulation as our high-protein diet; protein sources consisted of wheat germ (11.1% wet weight) and casein (2.3% wet weight). Rossiter (1987) showed that elimination of casein reduces pupal weights and prolongs development times for gypsy moths, so we used the same approach to produce a low-protein diet. In preliminary studies, we assessed the effects of juglone at levels of 0.1 and 0.2% (wet weight), which correspond to levels in fresh black walnut leaves (Hedin et al., 1979). Larvae were reared from egg hatch on the juglone diets and experienced excessively high mortality as second and third instars. Because our interests were in the sublethal effects of juglone, we lowered concentrations in our diets 10-fold. Our experimental design consisted of a 2 × 3 factorial with three levels of juglone (0, 0.01, and 0.02% wet weight) for each of two levels of protein. (Juglone was purchased from Sigma Chemical Co., St. Louis, Missouri; to prevent degradation, it was incorporated after diets were autoclaved.)

Bioassays. We conducted two types of bioassays to assess the impact of protein and juglone on gypsy moth performance. In the first, hereafter referred to as "survival trials," larvae were reared on the test diets from egg hatch to pupation, and survival rates, development times, and pupal weights were recorded as performance parameters. The second bioassay consisted of fourth-instar feeding trials, which allowed for calculation of standard nutritional indices. For both bioassays, larvae from each egg mass used were distributed across the treatments to equalize potential maternal or genetic effects.

Survival trials were initiated with six replicates of eight larvae for each of the six treatments. Larvae were contained through the third stadium in 1-oz

plastic cups, and thereafter in 5-oz plastic cups supplied with cubes of artificial diet. Diet was replaced and mortality rates recorded at three-day intervals for instars 1–4; thereafter cups were inspected daily for dead larvae and new pupae. We determined survival rates and development times from egg hatch to pupation. We also recorded pupal weight and sex three days after pupation.

For the fourth-instar feeding trials, larvae from each of eight egg masses were reared on each of the six experimental diets in groups of six to eight. Newly molted fourth instars (one per replicate) were placed into 1-oz cups containing a cube of one of the diets. Cups were cleaned and food replenished daily for the duration of the fourth stadium. At the end of each trial, we froze the larva, then dried (50°C) and weighed the larva, frass, and uneaten food. We estimated the dry weight of larvae at the onset of the trials based on proportion dry weights calculated from 14 newly molted fourth instars.

Enzyme Assays. Larvae to be used for enzyme preparations were reared on high- or low-protein diets, without juglone, for stadia 1–3. As new fourth instars, larvae were then reassigned to diets containing 0, 0.01, or 0.02% juglone, and the same level of protein as fed previously. Midguts (approximately 15 per replicate) were dissected from 2- to 4-day-old fifth instars, cleaned in 0.2 M potassium phosphate buffer (pH 7.8, with 1 mM EDTA), and homogenized by 10 strokes in a Ten Broeck tissue grinder. The homogenate was centrifuged at 10,000*g* (10 min) to remove cellular and mitochondrial debris; the supernatant was then centrifuged at 100,000*g* (60 min) to separate soluble (cytosolic) and microsomal (membrane-bound) enzymes. We resuspended the microsomal fractions in glycerol buffer (Brattsten, 1987) and flash froze the soluble and microsomal fractions in liquid nitrogen prior to storage at −70°C. All procedures were conducted at 0–4°C.

We conducted assays of four different enzyme systems involved in detoxification of xenobiotics in insects, including polysubstrate monooxygenase, esterase, quinone (carbonyl) reductase, and glutathione transferase. All assays were optimized with respect to enzyme concentration, incubation time, and buffer pH and conducted in duplicate or triplicate for each enzyme replicate. We calculated enzyme activities relative to protein concentrations, determined via a modified Folin–phenol procedure (Schacterle and Pollack, 1973).

Polysubstrate monooxygenase (PSMO) activity of gypsy moth larvae has been assessed frequently by the cytochrome *c* reductase and NADPH oxidation assays (Ahmad and Forgash, 1973, 1975, 1978; Sheppard and Friedman, 1989). The cytochrome *c* reductase assay measures production of reduced cytochrome *c* with time. The procedure we used was based on that of Brattsten et al. (1980). Each 1-ml assay solution contained 12–60 μg microsomal protein, 200 μl cytochrome *c* (4 mg/ml in phosphate buffer), 100 μl NADPH (1 mM in phosphate buffer), and 0.1 M potassium phosphate buffer (pH 8.0). Reduction of cytochrome *c* was measured as the increase in absorbance (550 nm) over 1 min

versus a blank solution that lacked the enzyme. A value of 27.6/mM/cm was used as the extinction coefficient of reduced cytochrome *c* (Margoliash and Frohwirt, 1959).

The NADPH oxidation assay measures endogenous oxidation of NADPH to NADP, in the absence of a cytochrome P-450 substrate. Our assay was conducted in a 250-μl incubation system, consisting of 0.1 M potassium phosphate buffer (pH 7.6), and 100–200 μg microsomal protein. The reaction was initiated with addition of 25 nmol NADPH to the sample cuvette. We monitored decrease in absorbance at 340 nm during the time interval 90–180 sec after addition of NADPH. (Nonlinear reaction rates precluded data acquisition immediately following addition of NADPH.) An extinction coefficient of 6.22/mM/cm (Segel, 1976) was used to calculate specific activity. We were unable to obtain NADPH oxidation data for larvae from two of the diet treatments. These microsomal preparations were used in an attempt to measure *N*-demethylation activity, which was unsuccessful because of poor reproducibility with the exceedingly low enzyme activities encountered.

We measured soluble esterase activity by the 1-naphthylacetate assay (Brattsten, 1987). Assay solutions contained 0.10–0.30 μg protein and sodium phosphate buffer (0.05 M, pH 8.0) to a total volume of 495 μl. Reactions were initiated by addition of 5 μl 1-naphthylacetate (50 mM in ETOH), incubated for 10 min (32°C), and terminated by addition of 1 ml of fast blue B solution (fast blue B and sodium dodecylsulfate at 0.2 and 7.5 mg/ml H_2O, respectively). After 10 min for color development, absorbances (600 nm) were read and converted to concentrations of 1-naphthol using a standard curve.

Quinone reductase (QR) activity of soluble and microsomal fractions was determined by the juglone-dependent NADPH oxidation method (Yu, 1987). Enzyme source (80–130 μg protein), 160 μl NADPH (2.5 mM in phosphate buffer), and sodium phosphate buffer (0.1 M, pH 8.0) were mixed to a volume of 2 ml. From that solution, 995 μl were placed into a sample cuvette, and the remainder into a reference cuvette. We added 5 μl juglone (10 mM in methyl cellosolve) to the sample cuvette to initiate the reaction. NADPH oxidation was quantified as the decrease in absorbance (340 nm) over several minutes. This assay system automatically cancels endogenous NADPH oxidation because enzymes and NADPH occur in both the sample and reference cuvettes. We established that the observed activity was due to a reductase rather than a microsomal oxidase by comparing QR activities between microsomal solutions treated and untreated with carbon monoxide (Yu, 1987; Lindroth, 1989a).

Glutathione transferase (GT) activity of soluble fractions was determined as the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with glutathione as described by Lindroth (1989b). Incubation mixtures contained 10–30 μg protein, 50 μl glutathione (50 mM in phosphate buffer), and potassium phosphate buffer (.1 M, pH 8.2) to a total of 975 μl. Reactions were initiated by addition

of 25 μl CDNB (40 mM in methyl cellosolve). GT activity was measured as the increase in absorbance (340 nm) over several minutes. We used an extinction coefficient of 9.6/mM/cm (Cohen, 1986) to calculate specific activity.

Statistics. Statistical analyses were performed with SAS statistical software. To assess potential effects of protein, juglone, and their interaction, data from the survival trials, feeding trials, and enzyme assays were analyzed by two-way ANOVA. In the survival trials, individual replicates were represented by up to eight larvae. Data on pupal weights were pooled, by sex, within each replicate. Proportional data (survival rate, approximate digestibility, and conversion efficiencies) were transformed (arcsin $\sqrt{y}$) prior to analysis.

RESULTS

Survival Trials. Survival rates of gypsy moths, from egg hatch to pupation, were unaffected by dietary protein levels, but strongly affected by juglone (Table 1). Juglone at the 0.01% level did not significantly reduce survival, but at 0.02% reduced survival of larvae on the high- and low-protein diets by 40% and 64%, respectively. Although larvae fed juglone tended to survive less well on the low-protein diet than on the high-protein diet, the protein × juglone interaction was not statistically significant.

Pupal weights were influenced by both protein and juglone (Table 1). Low-protein diets without juglone reduced weights of males and females by 19% and 31%, respectively. Juglone reduced weights of male pupae by up to 45%, and did so independently of protein content. In contrast, dietary protein did alter the effect of juglone on females, reducing weights of pupae from high-protein diets by up to 48%, but of pupae from low-protein diets only 25%.

Protein and juglone both influenced larval development times (Table 1). In comparison with larvae fed the high-protein diet, those fed the low-protein diet required an additional four to five days to complete development. Juglone greatly prolonged development times, and more so for females than males. Protein concentrations did not influence effects of juglone on larval duration.

Feeding Trials. Results from the fourth-instar feeding trials also reveal effects of dietary protein and juglone on larval performance (Table 2). Juglone, but not low levels of protein, prolonged fourth-stadium duration. The lack of an effect of low protein contrasts with the effect observed for larval development time (Table 1) and indicates that consequences of low levels of protein are more pronounced for larval stadia earlier or later than the fourth. Similarly, juglone, but not low levels of protein, reduced larval growth rates. Juglone at 0.01% reduced growth about 40%, and at 0.02% an additional 13%. Larvae increased consumption rates when fed low-protein diets, but markedly reduced consumption rates when fed juglone. Diet digestibilities (ADs) were higher for the high-

TABLE 1. EFFECTS OF PROTEIN AND JUGLONE ON SURVIVAL RATES, PUPAL WEIGHTS, AND DEVELOPMENT TIMES OF GYPSY MOTHS[a]

Diet	Survival	Pupal weight (mg)		Duration (days)	
		Males	Females	Males	Females
High protein					
0.00% juglone	89.6 ± 12.3 (6)	733 ± 23 (5)	2720 ± 148 (6)	30.2 ± 0.4 (5)	34.6 ± 0.5 (6)
0.01% juglone	95.0 ± 11.2 (5)	573 ± 31 (5)	2171 ± 72 (5)	39.8 ± 1.7 (5)	47.9 ± 1.5 (5)
0.02% juglone	54.2 ± 23.3 (6)	496 ± 46 (4)	1364 ± 89 (5)	46.4 ± 2.9 (4)	55.4 ± 1.2 (5)
Low protein					
0.00% juglone	97.9 ± 5.1 (6)	592 ± 11 (6)	1873 ± 80 (6)	33.9 ± 0.6 (6)	39.5 ± 0.3 (6)
0.01% juglone	83.3 ± 15.1 (6)	418 ± 15 (6)	1385 ± 132 (5)	45.8 ± 0.7 (6)	56.5 ± 1.2 (5)
0.02% juglone	35.4 ± 22.9 (6)	325 ± 30 (3)	1428 ± 135 (4)	51.8 ± 1.3 (3)	60.9 ± 2.5 (4)
P values					
Protein effect	0.310	0.004	0.002	0.009	<0.001
Juglone effect	<0.001	0.002	0.002	<0.001	<0.001
Protein × juglone interaction	0.092	0.966	0.036	0.475	0.754

[a] $\bar{X} \pm 1$ SE; sample size in parentheses.

TABLE 2. EFFECTS OF PROTEIN AND JUGLONE ON NUTRITIONAL INDICES OF FOURTH-INSTAR GYPSY MOTHS[a]

Diet	Duration (days)	RGR (mg/mg/day)	RCR (mg/mg/day)	AD (%)	ECD (%)	ECI (%)
High protein						
0.00% juglone (7)	5.0 ± 0.3	0.23 ± 0.01	0.71 ± 0.02	65.7 ± 1.0	49.4 ± 0.5	32.5 ± 0.6
0.01% juglone (6)	7.6 ± 0.6	0.14 ± 0.02	0.45 ± 0.04	55.2 ± 1.3	56.7 ± 1.5	31.2 ± 0.7
0.02% juglone (6)	8.2 ± 0.3	0.11 ± 0.02	0.39 ± 0.04	51.3 ± 2.1	51.4 ± 4.1	26.7 ± 2.7
Low protein						
0.00% juglone (8)	5.1 ± 0.2	0.24 ± 0.01	0.85 ± 0.03	55.6 ± 0.6	50.3 ± 1.2	27.9 ± 0.5
0.01% juglone (6)	7.4 ± 0.4	0.14 ± 0.00	0.51 ± 0.02	53.6 ± 1.8	51.0 ± 3.7	27.0 ± 1.4
0.02% juglone (5)	7.8 ± 0.4	0.11 ± 0.01	0.44 ± 0.03	48.8 ± 2.3	55.3 ± 5.3	26.5 ± 1.3
P values						
Protein effect	0.649	0.916	0.004	<0.001	0.905	0.012
Juglone effect	<0.001	<0.001	<0.001	<0.001	0.334	0.046
Protein × juglone interaction	0.767	0.815	0.308	0.008	0.261	0.235

[a] $\bar{X} \pm 1$ SE; sample size in parentheses. RGR = relative growth rate. RCR = relative consumption rate. AD = approximate digestibility. ECD = efficiency of conversion of digested food. ECI = efficiency of conversion of ingested food.

protein diets than low-protein diets, and in all cases were reduced with addition of juglone. Juglone decreased ADs to a greater extent in animals fed high-protein diets than in those fed low-protein diets, resulting in a significant protein × juglone interaction term. Efficiencies of conversion of digested food (ECDs) were unaffected by either protein or juglone. Efficiencies of conversion of ingested food (ECIs) were higher in larvae fed high-proetin diets, and lower in larvae fed juglone diets, largely reflecting trends in AD values. These data indicate that dietary juglone suppressed growth primarily by reducing consumption rates and secondarily by reducing food digestibility.

Enzyme Assays. Cytochrome *c* reductase and NADPH oxidation activities indicate the capacity of enzymes to oxidatively metabolize compounds. We found that neither dietary protein nor juglone influenced oxidative activity in fifth instars (Table 3).

General esterase activity (soluble enzymes) was influenced by both dietary protein and juglone. Activity was 1.4-fold higher in larvae fed the low-protein diet than in those fed the high-protein diet (Table 3). Moreover, in all but one case, esterase activity was induced in larvae fed juglone. The largest effect was a 38% increase occurring in larvae fed the low-protein, 0.01% juglone diet. The single exception occurred in larvae fed the low-protein, 0.02% juglone diet, which exhibited no significant increase in esterase activity.

Soluble quinone reductase activity was generally higher in larvae fed low-protein diets than in larvae fed high protein diets (Table 3). In contrast, microsomal QR activity was unaffected by protein level. Neither soluble nor microsomal QR activities were influenced by consumption of juglone by gypsy moth larvae. Microsomal QR activities were similar to or slightly higher than soluble QR activities, per unit protein in the enzyme preparations. Soluble fractions, however, contained 8–12 times as much protein as did microsomal fractions. Thus, soluble enzymes accounted for a much higher proportion of total QR activity than did microsomal enzymes.

Glutathione transferase activity was influenced by dietary protein levels, ranging from 12% to 36% higher in high-protein diets than in the corresponding low-protein diets (Table 3). Juglone, however, did not significantly affect larval GT activity.

DISCUSSION

Foliar protein is of singular importance in determining the quality of plant tissues for insect herbivores (Mattson, 1980; Scriber and Slansky, 1981; White, 1984). This is particularly true of tree-feeders such as gypsy moths, because tree leaves generally contain low levels of protein. Results from this study show that inadequate dietary protein may affect several performance parameters in gypsy moths. As also observed by Rossiter (1987), elimination of casein reduced

TABLE 3. EFFECTS OF PROTEIN AND JUGLONE ON MIDGUT ENZYME ACTIVITIES OF FIFTH-INSTAR GYPSY MOTHS[a]

Diet	Cytochrome *c* reductase	NADPH oxidation	Esterase	Quinone reductase		Glutathione transferase
				Soluble	Microsomal	
High protein						
0.00% juglone	105.4 ± 5.6	1.20 ± 0.12[b]	2197 ± 149	25.8 ± 2.3	34.7 ± 3.1	672.4 ± 34.8
0.01% juglone	88.2 ± 18.1	1.11 ± 0.10	3217 ± 227	32.2 ± 4.3	31.1 ± 7.2	715.1 ± 58.2
0.02% juglone	133.1 ± 15.8		2997 ± 77	20.3 ± 2.2	49.7 ± 4.9	602.0 ± 18.2
Low protein						
0.00% juglone	111.9 ± 30.1		3104 ± 189	34.4 ± 2.8	40.3 ± 12.6	599.1 ± 48.5
0.01% juglone	94.4 ± 29.4	1.17 ± 0.13	4299 ± 272	32.3 ± 5.6	32.2 ± 10.8	526.6 ± 58.4
0.02% juglone	118.0 ± 22.3	1.12 ± 0.26	3312 ± 248	32.4 ± 3.4	37.7 ± 7.6	475.2 ± 51.4
P values						
Protein effect	0.965	0.964	<0.001	0.029	0.804	0.003
Juglone effect	0.312	0.915	<0.001	0.270	0.370	0.107
Protein × juglone interaction	0.853		0.116	0.253	0.556	0.485

[a] $\bar{X} \pm 1$ SE; sample size for each treatment is 5. All specific activities shown as nmol/min/mg protein.
[b] $N = 4$ for this treatment.

pupal weights of males and females and prolonged their development times. Our observations indicated that prolonged development occurred in early larval stadia; no effect was seen with fourth instars. We also found no effect of low protein on relative growth rates of fourth instars. Relative rates, however, obscure some important differences. At the onset of the feeding trials, larvae reared on the high-protein diet (with no juglone) averaged 46% heavier than those reared on the low-protein diet, and average (not relative) growth of larvae in the two treatments differed by 34% during the fourth stadium. These data also suggest that young instars are particularly susceptible to low-protein diets. Older larvae appear to compensate for low levels of dietary protein by increasing consumption rates. Larvae fed high-protein diets digested a higher proportion of their food than did animals fed the low-protein diet. This difference is probably a consequence of both the high digestibility of casein and reduced efficiency of digestion in larvae with high consumption rates. Whether protein effects on gypsy moth performance were due to insufficient protein quantity or quality is not known, although the latter possibility is as likely as the former. Casein apparently complements the amino acid composition of wheat germ protein (R.A. Bell, A.R.S., Beltsville, Maryland, personal communication). Broadway and Duffey (1988) showed that, of several characteristics of protein quality, amino acid profile was the most important in determining the quality of dietary protein to *Spodoptera exigua.*

Although almost nothing is known about the effect of dietary protein on the activity of detoxification enzymes in insects, considerable information is available from studies with other animals. Inadequate dietary protein may reduce detoxification enzyme activity because fewer amino acids are available for biosynthesis of enzymes and cofactors. For example, diets deficient in quality or quantity of protein often cause reductions in cytochrome P-450 (the terminal oxidase of the PSMO system) and cytochrome P-450 reductase, with subsequent impairment of microsomal oxidase activity (Parke and Ioannides, 1981; Boyd and Campbell, 1983). Induction of microsomal enzyme activity may be inhibited also. Effects are not always consistent, however. Protein-deficient diets are known to increase, decrease, or have no effect on conjugation reactions of detoxification enzymes. We found no effect of low protein on activities of PSMO or microsomal QR enzymes. Interestingly, low-protein diets prompted an increase in soluble esterase and QR enzyme activities; the reason for these responses is unclear. Finally, these diets reduced glutathione transferase activities, perhaps because of reduced synthesis of the transferase enzyme itself. Low-protein diets also may have reduced transferase activities in vivo because of decreased availability of glutathione, a tripeptide cosubstrate. This possibility would not have been observed in the in vitro enzyme assay, because glutathione is added to the incubation mixture. A marked effect of low protein on enzyme activities may not have occurred because older larvae may not have been seriously protein stressed.

Although juglone is a well-known allelopathic agent (Ponder, 1987), its effects on phytophagous insects have received little study. Gilbert et al. (1967) found that juglone isolated from *Carya ovata* deterred feeding by the bark beetle, *Scolytus multistriatus*. Yu (1987) demonstrated that juglone decreased growth and increased mortality in the fall armyworm, *Spodoptera frugiperda*. Our results show that at doses lower than those measured in black walnut leaves (Hedin et al., 1979), juglone markedly increases mortality, decreases growth, and prolongs development times of gypsy moths. These effects are due primarily to reduced consumption rates and secondarily to reduced approximate digestibilities, but not to altered conversion efficiencies of digested food (ECDs). Effects of juglone were mostly independent from those of protein. Low-protein diets did augment the effects of juglone on pupal weights of females and digestion efficiencies of fourth instars.

The specific mode of action of juglone is unknown, but several possibilities exist (Norris, 1988). First, both the carbonyl and hydroxyl groups of juglone and related quinonoid diketones exhibit hydrogen bonding. Second, because of their α,β unsaturation, these compounds readily undergo nucleophilic 1,4 (Michael) additions with a variety of nucleophiles (e.g., sulfhydryl, amino, and hydroxyl groups). Thus, juglone could conceivably interfere with digestion by binding (via hydrogen or covalent bonds) to digestive enzymes, thereby reducing approximate digestibilities as observed in our study. Alternatively, juglone could interfere with any of a host of other biochemical/physiological processes by binding to critical macromolecules.

Quinone reductase and glutathione transferase are the enzyme systems of particular relevance for detoxification of juglone by insects. Quinone reductase reduces juglone to 1,4,5-trihydroxynaphthalene in the fall armyworm, which performs poorly on juglone-containing diets (Yu, 1987). Microsomal QR activity of four lepidopteran species, none of which utilize species in the Juglandaceae, ranged from 35 to 51 nmol/min/mg protein (Yu, 1987). Gypsy moth QR activities are of comparable magnitude and are six-fold less than values determined for larvae of the luna moth (*Actias luna*), which perform quite well on species in the Juglandaceae (Lindroth, 1989a). Thus, low constitutive levels of QR activity and lack of an induced response may explain the inability of gypsy moths to grow and survive well on diets containing juglone.

Although no studies have addressed detoxification of juglone via glutathione transferases, that such a possibility likely occurs is indicated by the fact that glutathione transferases catalyze the metabolism of many compounds containing α,β-unsaturated carbonyls (Wadleigh and Yu, 1987). Given that juglone is one such compound and that quinones are very reactive toward sulfhydryl groups in particular (Norris 1988), conjugation with glutathione (a sulfhydryl-containing tripeptide) may be a particularly effective means for detoxifying juglone in insects. Indeed, GT activities in *Actias luna* are extraordinarily high

(Lindroth, 1989a). Glutathione transferase activities in gypsy moth larvae are comparable to those of several other Lepidoptera (Gunderson et al., 1986; Brattsten, 1987; Lindroth, 1989b). This moderate level of activity may be inadequate for substantial detoxification of juglone by gypsy moths. Our data suggest that whatever capacity larvae do have may be constrained further by low levels of dietary protein. Moreover, larvae fed low-protein diets exhibited a trend toward reduced GT activity with an increase in juglone concentration. Thus, particularly low GT activity was correlated with, and may have contributed to, the especially poor performance of larvae on the low-protein, 0.02% juglone diet.

That juglone is not oxidatively metabolized in gypsy moths is supported by our observations with carbon monoxide-treated microsomes. PSMO-mediated oxidation of juglone would contribute to NADPH oxidation in the quinone reductase assay. Administration of carbon monoxide, which inhibits PSMO activity, did not alter NADPH oxidation rates, indicating lack of monoxygenase activity.

Esterases are also unlikely to be involved in metabolism of juglone. Elevated esterase activity in larvae fed juglone is probably an example of induction of an enzyme system by a chemical that does not serve as a substrate for the enzyme.

In conclusion, this study showed that dietary protein and juglone, and their interactions, influence the performance of gypsy moth larvae. The effects of juglone are quite striking at very low doses, suggesting that this compound or structural analogs in members of the Juglandaceae may be responsible for the moderate to poor acceptability of these species to gypsy moths. Poor performance of larvae fed juglone may be linked to the moderate to low activities of the major juglone-detoxifying systems in these insects. Our prediction that low-protein diets would potentiate the toxicity of juglone via decreased activity of detoxification enzymes was partially supported, in that larvae fed low levels of protein exhibited significant reductions in GT activity.

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EVIDENCE FOR TRAIL-PHEROMONE PRECURSOR IN TERMITE *Reticulitermes speratus* (KOLBE) (RHINOTERMITIDAE: ISOPTERA)

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Abstract—Whole body extracts of the termite, *Reticulitermes speratus*, were subjected to various chemical operations and bioassays to examine the presence of trail-pheromone precursor. Fractions that mainly contained fatty acid esters were obtained from hexane extracts by means of silica gel column chromatography. Trail-following activity of the fractions was activated by alkaline hydrolysis, while the original fractions did not show any conspicuous activity. Bioassay showed that the activity of hydrolyzed product was approximately 20 times as high as the original hexane extract. This suggests that the precursor candidate could be stored in termite bodies as an esterified form. Chemical analyses revealed that the complete structure of the hydrolyzed product was coincident with that of the native pheromone ((*Z*,*Z*,*E*)-3,6,8-dodecatrien-1-ol).

Key Words—Subterranean termite, *Reticulitermes speratus*, Rhinotermitiade, Isoptera, trail pheromone, precursor candidate, alkaline hydrolysis, isolation, (*Z*,*Z*,*E*)-3,6,8-dodecatrien-1-ol.

INTRODUCTION

The presence of a termite trail pheromone was first demonstrated by Stuart (1961, 1963, 1969). Moore (1966, 1969) was the first to isolate a trail pheromone from whole-body extracts of *Nasutitermes exitiosus* (Hill) (Nasutitermi-

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tinae: Termitidae) and identified it as the unsaturated diterpene hydrocarbon $C_{20}H_{32}$ (neocembrene-A) (Birch et al., 1972).

Matsumura et al. (1968, 1969) also isolated a trail pheromone from whole-body extracts of *Reticulitermes virginicus* (Banks) (Rhinotermitidae). The amount of isolated pheromone was too small to allow determination of the complete chemical structure. On the other hand, they found that a substance isolated from wood decayed by the fungus, *Gloeophyllum trabeum* (Pers. ex. Fr.), was similar to the trail pheromone of *R. virginicus* chemically and identified it as (*Z,Z,E*)-3,6,8-dodecatrien-1-ol (DTE-OH). They finally concluded that the chemical was identical with the trail pheromone of *R. virginicus* (Tai et al., 1969).

More recently, Tai et al. (1971), Ritter and Persoons (1975), Ritter et al. (1977), and Prestwich et al. (1984) synthesized analogs of the trail pheromone in order to assess the relationship between the trail-following behavior and the stereochemical structure of such substances. They and others demonstrated that many of the analogs could induce trail-following behavior (Tai et al., 1971; Matsumura et al., 1972; Kaib et al., 1982).

DTE-OH also elicited trail-following behavior in other species within the genus *Reticulitermes*, e.g., *R. flavipes* (Kollar), *R. hesperus* Banks, *R. lucifugus santonensis* Feytaud, *R. tibialis* Banks, *R. speratus* (Kolbe), as well as *Coptotermes formosanus* Shiraki, also in the family Rhinotermitidae (Matsumura et al., 1972; Ritter and Persoons, 1975; Howard et al., 1976; Yamaoka et al., 1987; Grace, 1987). The trail-following substance thus was shown to exhibit weak species specifically. It is possible that DTE-OH is a common trail pheromone within the family Rhinotermitidae.

We isolated and identified the trail pheromone of *R. speratus* as DTE-OH, which was exactly the same structure as that of *R. virginicus* (Yamaoka et al., 1987). On the basis of the small amount of trail pheromone extracted from the termites, each individual did not seem to induce trail-following behavior over a long distance in the natural habitat. Therefore, it was hypothesized that the pheromone might be stored in the body of the termite in the form of a precursor and secreted as required transformation from the precursor.

The main purpose of the present investigation was to find the precursor candidate of the trail pheromone of *R. speratus* by chemical techniques and bioassays.

METHODS AND MATERIALS

Insects. Test animals used in the present investigation were obtained from two colonies of *Reticulitermes speratus* collected from termite-attacked timber on the campus of Kyoto Institute of Technology. Individual termites were care-

fully taken out of the timber for extraction. The remaining termites from one colony were kept at 20 ± 2°C and 50 ± 5% relative humidity with occasional moistening in the laboratory over three months until they were subjected to bioassay. In this investigation, we used undifferentiated *R. speratus* pseudergates that were older than the third instar as determined by size of the termite body (hereinafter referred to as worker). Test individuals for extraction were placed on moistened filter paper for about 5 hr before extraction. The termites used for bioassays were held in a Petri dish with moistened filter paper for 1 hr before the test.

Chemicals. (Z,Z)-3,6-Dodecadien-1-ol and (Z)-3-dodecen-1-ol were supplied by Dr. G. D. Prestwich (State University of New York at Stony Brook). Isomers of 6,8-dodecadien-1-ol were supplied by Dr. T. Ando (Tokyo University of Agriculture and Technology). All other reagents were purchased from Nakarai Chemicals Limited (Kyoto, Japan).

Apparatus. The high-performance liquid chromatograph (HPLC) was a model LC-5A (Shimadzu) pump connected to a model UVIDEC-100II detector (set at 234 nm; Jasco, Tokyo, Japan), equipped with both a Cosmosil 10SL guard column (5 cm × 4.6 cm) ID, silica gel, particle size 10 μm; Nakarai) and a Cosmosil 5SL column (25 cm × 4.6 cm ID, particle size 5 μm).

Gas chromatography (GC) employed a YANACO model G180PF (Yanaco, Kyoto, Japan), equipped with FID detector. The nonpolar column was a wide-bore capillary column (10 m × 0.53 mm ID; Hewlett-Packard (HP), Avondale, Pennsylvania) coated with methyl silicone (2.5 μm). The polar column was a glass column (1.5 m × 3 mm ID) packed with 5% Carbowax 20 M-W-HP (100–120 mesh; HP).

The gas chromatograph–mass spectrometer (GC-MS) was a model 5970 gas chromatograph (HP) combined with a model M-80B mass spectrometer equipped with a model 0101 on-line data system (Hitachi, Ibaraki, Japan). A fused-silica capillary column (Shimadzu CBP 20M, 25 m × 0.25 mm ID) also was used with this system.

Bioassay. A modification of the Prestwich et al. (1984) open-field bioassay (Howard et al., 1976) was used to establish threshold response levels for trail-following as described elsewhere (Tokoro et al., 1989). When three or more of the nine test worker termites showed the complete trail-following action, the sample was considered to have elicited a positive response. Ten-fold-dilution series and more finely diluted solutions of each sample were employed to determine the "minimum effective worker equivalent" (MEWE, worker equivalent/15-cm sample trail). The relative strength of trail-following activity was compared on the basis of the MEWE. Bioassays were carried out at 20 ± 2°C, 50 ± 5% relative humidity and under overhead fluorescent lighting. No termite was used for more than one bioassay in 24 hr, and one worker was used per test trail.

Extraction. The worker termites (approximately 146 g, ca. 97,000 in number) were then soaked in a liter of *n*-hexane for three days without homogenization, and the extracts were subsequently filtered and stored at −20°C until the next step.

Isolation and Activation. The hexane extracts were dried with anhydrous Na_2SO_4 and concentrated by rotary vacuum evaporator to obtain the yellowish crude lipid (ca. 3 g). This crude lipid was first fractionated by silica-gel column chromatography with *n*-hexane (Hx)–ethyl acetate (EtOAc) and CH_3OH eluants.

Fractions that did not show any trail-following activity in the initial test were subjected to various kinds of chemical reaction, because it was assumed that a precursor could exist. Active trail-following behavior was produced for the 5% and 10% EtOAc–Hx fractions by hydrolysis with 2% KOH–CH_3OH.

The yellowish crude lipids (ca. 1.3 g) obtained from these fractions were dissolved in dry diethyl ether and hydrolyzed with 2% KOH–CH_3OH for 10 hr at room temperature. After hydrolysis, the reaction mixtures were separated with diethyl ether and water in a separatory funnel. The ether layer was washed once with 1 N HCl and twice with distilled water and dried with anahydrous Na_2SO_4.

Subsequently, this activated fraction (ether layer, 155 mg) was purified by silica-gel column chromatography, followed by 20% (w/w) $AgNO_3$ silica-gel column chromatography HPLC as described elsewhere (Tokoro et al., 1989).

The final purification step was carried out by GC using nonpolar and polar columns. Analytical conditions for each column were as follows: nonpolar column, 140°C (injection 250°C), carrier gas N_2 (40 cm/sec); polar column, 180°C (injection 250°C), carrier gas N_2 (25 ml/min). In both analyses, the outlet gas for each peak was isolated and the trail-following activity in response to it was determined.

Identification. The active GC peak was analyzed by capillary GC-MS. Structure determination of the hydrolysis product was attempted by means of capillary GC-MS and capillary GC-FTIR analysis combined with microscale chemical reactions, as described previously (Yamaoka et al., 1987).

RESULTS AND DISCUSSION

Results of trail-following activity bioassays with crude hexane extracts are shown in Table 1. Early scientists have shown that trail-following activity is induced by the trail pheromone within a limited range of worker equivalents (WE) (Moore, 1966; Hall and Traniello, 1985). Termites show little or no response outside of the range. *R. speratus* was no exception.

As shown in Table 1, the hexane extracts exhibited remarkable activities

TABLE 1. TRAIL-FOLLOWING BIOASSAYS WITH CRUDE HEXANE EXTRACTS[a]

	Quantity (WE/15 cm)[b]						
	1000	100	10	1	0.1	0.01	0.001
Percentage following[c]	10.0	80.0	90.0	93.3	63.3	10.0	0.0

[a]Bioassays were modified open-field bioassays (Howard et al., 1976) with 5 μl of test solution applied to a 15-cm circle.
[b]Worker equivalent of 5 μl test solution.
[c]Percentage of workers trail following per 30 workers.

ranging from 100 to 0.1 WE/15 cm. The MEWE was thus identified as 0.1 WE/15 cm. In the preliminary experiments, the extracts of intestinal contents of test workers showed no trail-following activity. After extracting with hexane, the corpses were homogenized with diethyl ether and measured for trail-following activity. The activity was less than one-fiftieth of the primary hexane extracts.

The results of the bioassays with the hexane extract indicated that a 0.1 WE extract induced trail-following over a 15-cm trail, so a worker termite could only produce a 1–5-m-long secretion of the trail pheromone (MacFarlane, 1983) was not clear in rhinotermitid termite (Grace et al., 1988), the quantity of trail pheromone in a worker seems to be very small. This phenomenon led to the assumption that the pheromone is stored in the body in the form of precursor that is secreted as required following conversion to the active form.

The native trail pheromone was eluted mainly in the range of 15–20% EtOAc–Hx on silica-gel column chromatography (Table 2). Preliminary experiments, which were intended to investigate the possible existence in the extract of a pheromone precursor, showed that fractions eluted with 5–10% EtOAc–Hx were activated both by hydrolysis with 2% KOH–CH_3OH and by reduction with $LiAlH_4$. Since the fraction mainly contained fatty-acid esters, it was suspected that the precursor candidate would be stored as an esterified pheromone. The activity of the newly activated substances were about 20 times that of the native pheromone by weight (Table 2).

Hydrolysis reaction was subsequently employed because it is milder. After separation with diethyl ether and water, the ether layer showed high activity (0.005 MEWE). When the water layer was acidified with 1 N HCl solution, a quantity of free fatty acids that were supposed to derive from triglyceride by hydrolysis was separated.

Behavior of the hydrolyzed product on these chromatographies closely corresponded to that of the native pheromone (Table 2). In addition, the capillary

TABLE 2. TRAIL-FOLLOWING BIOASSAY AFTER SILICA-GEL COLUMN CHROMATOGRAPHY

Fraction (%)	Activity as MEWE (WE/15 cm)[a]		
	Hexane extracts		Activated fractions[b]
	Native	After hydrolysis	
Whole	0.1	0.005	0.005
0[c]	[d]	[d]	[d]
5	[d]	0.005	[d]
10	[d]	0.01	[d]
15	0.1	(0.1)[e]	0.006
20	1	(1)	0.01
100	[d]	[d]	1
CH_3OH	[d]	[d]	[d]

[a]The activity was estimated by a modified open-field bioassay. When three or more of the nine workers followed the 15-cm trail, the sample was designated positive. Tenfold dilution series and more highly diluted solutions of each sample were employed to determine the "minimum effective worker equivalent" (WE/15-cm trail).
[b]This fraction consist of 5% and 10% fractions of the hexane extracts after hydrolysis.
[c]Percentage by volume of EtOAc–Hx (%) as eluants.
[d]No response or insufficient response for trail following.
[e]The values in parentheses are native activity.

GC-MS data of the hydrolyzed product coincided well with that of the native pheromone, exhibiting the same retention time on capillary GC and the same pattern in the mass spectrum (Figure 1).

Finally, we determined the complete chemical structure of the hydrolyzed product as (*Z,Z,E*)-3,6,8-dodecatrien-1-ol by means of instrumental analyses (Yamaoka et al., 1987). It was identical with the native trail pheromone. The amount of pheromone isolated was estimated on the basis of GC analysis to be about 300 ng for the native pheromone and about 5 μg for the hydrolyzed product. The quantity of the isolated native pheromone was nearly equivalent to that of the trail pheromone in *R. virginicus* (Matsumura et al., 1968; 1969).

Further investigation is needed in order to confirm whether this precursor does exist. The possibility will remain that DTE-OH is not synthesized by termites until this hypothesis is tested by rearing termites on food that is free of microflora and microfauna. The present results, however, suggest that DTE-OH is stored as an ester prior to its transformation to pheromone. It is, therefore, of great scientific interest to specify the storing organ of the precursor candidate.

Trials to determine the chemical structure of the acid moiety that was combined with the alcohol moiety of the trail pheromone suggested that the precur-

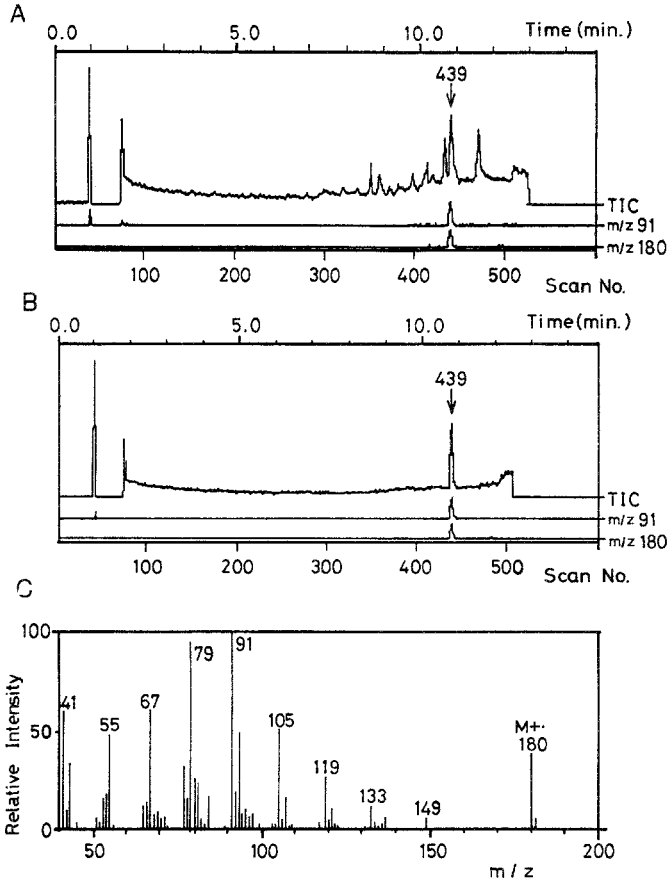


FIG. 1. Results of capillary GC-MS. Total ion chromatograms and mass chromatograms of the native active fractions (trail pheromone) (A) and of the newly activated fractions by hydrolysis (B); The arrows indicate the active peaks: scan 439. (C) Mass spectrum of the active peak scan 439 (B).

sor candidate did not seem to consist of only one component. Investigation of the variation of the precursor candidate would contribute to the taxonomy of the family Rhinotermitidae, as did defense compounds (Prestwich, 1984).

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EFFECTS OF MIXTURES OF PHENOLIC ACIDS ON PHOSPHORUS UPTAKE BY CUCUMBER SEEDLINGS[1]

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Abstract—To determine how individual phenolic acids in a mixture might affect phosphorus (P) uptake, 15-day-old cucumber seedlings grown in solution culture were treated with ferulic, vanillic, *p*-coumaric, or equimolar mixtures of these phenolic acids. Phenolic acid and P uptake were determined by solution depletion. The joint action of the mixtures of these phenolic acids on P uptake was primarily additive. Thus, as the number of phenolic acids increased in the mixture, the concentrations of the individual phenolic acids in the mixture required to bring about a given response declined. Seedling uptake of individual phenolic acids from solution mixtures of phenolic acids was reduced when compared to the uptake of phenolic acids from single phenolic acid solutions. The magnitude of the reduction varied with phenolic acid and concentration. The dose required for 50% inhibition of P uptake was approximately two to three times higher for vanillic acid (6.73 mM) than for ferulic (2.27 mM) and *p*-coumaric acids (3.00 mM) when dose was based on the initial treatment concentrations. The dose required for 50% inhibition of P uptake was not significantly different for the three phenolic acids (42 $\pm$ 5 μmol/g root fresh weight) when dose was based on phenolic acid uptake. Potential reasons for these differences are discussed.

Key Words—Joint action analysis, phenolic acid mixtures, phosphorus uptake, phenolic acid uptake, ferulic acid, vanillic acid, *p*-coumaric acid, *Cucumis sativus*.

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INTRODUCTION

Among the types of chemical compounds identified as allelopathic agents, simple phenolic acids including ferulic acid (FA), *p*-coumaric acid (*p*-CA), and vanillic acid (VA) are the most frequently cited in the literature (see Rice, 1984). Studies on the impact of these individual phenolic acids in nutrient culture suggest that the concentrations required for inhibition of germination, growth, and various plant functions are in most cases above 0.1 mM, which is well above concentrations in water extracts of soil (Whitehead et al., 1981). Allelopathic interactions in soils, however, are not a result of individual phenolic acids but a product of complex mixtures of phenolic acids, as well as other allelopathic compounds. The dynamic nature of phenolic acid mixtures in soils, furthermore, leads to considerable difficulty in predicting plant response, since the action of individual phenolic acids in mixtures can be additive, antagonistic, or synergistic (Rasmussen and Einhellig, 1977; Williams and Hoagland, 1982; Blum et al., 1984, 1985, 1989). The plant processes affected by phenolic acids relate to energy fixation, water relations, and/or plant nutrition. In the latter case, a number of researchers have shown that individual phenolic acids inhibit phosphorus (P) uptake by root sections and seedling roots (Glass, 1974a, 1975; McClure et al., 1978).

The objective of this study was to determine how individual phenolic acids in a mixture might affect P uptake. Concurrently, we also estimated the uptake of the individual phenolic acids from the mixture by seedlings.

METHODS AND MATERIALS

Plant Material. Seedlings of *Cucumis sativus* cv. Early Green Cluster (Wyatt Quarles Seed Company, Raleigh, North Carolina) were germinated in the dark at 30°C in trays containing vermiculite and full-strength Hoagland's solution (Hoagland and Arnon, 1950). Two days after sowing, the seedlings were placed under a light bank (140 $\mu E/m^2/sec$ for 13 hr) before being transferred to 120-ml jars (one seedling per jar) containing full-strength Hoagland's solution, which was aerated continuously. Seedlings were grown for 10 days under three light banks at room temperature (21–31°C). Nutrient solution (pH 5.5) was changed every five days. Deionized water was added daily.

Phenolic Acid Treatments. Phenolic acid treatments occurred when seedlings were 15 days from seeding. Twenty four hours prior to phenolic acid treatments, the nutrient solution was replaced with 0.5 mM $CaSO_4$ solution. Seedlings were then transferred into solutions (100 ml; one seedling per jar) containing 0.5 mM $CaSO_4$, 0.5 mM KH_2PO_4, and 0, 0.125, 0.25, 0.5, or 1 mM FA, VA, or p-CA, or equimolar mixtures of two and three of these acids.

The concentrations of each acid in the FA + VA, FA + *p*-CA, and VA + *p*-CA mixtures were 0.125, 0.25, or 0.5 mM. The concentrations of each acid in the three-way mixtures were 0.083, 0.167, or 0.333 mM.

Uptake. The uptake of the phenolic acids and phosphorus per unit of fresh weight of root was based on the depletion of these components from solution. The phenolic acids and P remaining 8 hr after the initial treatment were determined with a high-performance liquid chromatograph (Blum and Dalton, 1985) and spectrophotometer (Taussky and Shorr, 1953), respectively.

Data Analysis. All treatments (three replicates) were randomly distributed under three light banks ($N = 75$). Data were analyzed using the Statistical Analysis System procedures for analysis of variance, linear regression, and nonlinear regression (SAS Institute, 1988). Joint action analysis of data was according to Gerig et al. (1989). The model for joint action analysis allows for antagonistic, synergistic, and similar joint (additive) action of compounds. It also allows for differential potencies of the compounds. The uptake of P was modeled by the function

$$f(Z;\ \beta) = \begin{cases} \beta_1/(1 + \exp\{\beta_3[\ln(Z) - \beta_2]\}), & Z > 0 \\ \beta_1, & Z = 0 \end{cases}$$

where the effective concentration Z is defined by

$$Z = cFA + \beta_{41}\, cVA + \beta_{42}\, cpCA + 2\beta_{51}(\beta_{41}\, cFA\, cVA)^{1/2} + 2\beta_{52}(\beta_{42}\, cFA\, cpCA)^{1/2} + 2\beta_{53}(\beta_{41}\, \beta_{42}\, cVA\, cpCA)^{1/2}$$

and where *cFA*, *cVA* and *cpCA* denote the concentrations of FA, VA, and *p*-CA, respectively. The parameters of the model for this specific analysis are as follows: β_1 is the P uptake of control seedlings; β_2 is the natural log of the dose of FA required for 50% inhibition of P uptake [50% inhibition dose for VA and *p*-CA may be calculated by dividing the 50% dose of FA by the appropriate relative potency (β_4)]; β_3 is the rate at which the response curve descends; β_{4j} are the relative potencies of VA and *p*-CA expressed in terms of FA; β_{5j} are the coefficients of synergy (determine the degree of departure of a model from a model for similar joint action).

RESULTS

Phenolic Acid Uptake. Mean fresh weights of shoots and roots, and leaf areas of all cucumber seedlings were 2.88 ± 0.02 g (mean ± SE), 0.95 ± 0.01 g, and 59.55 ± 0.60 cm^2, respectively. Eight-hour uptake (micromoles per

gram of root fresh weight) of FA, VA, or *p*-CA by these 15-day-old seedlings increased as the initial treatment concentration increased (Table 1). The uptake of FA for the single phenolic acid treatments was approximately 1.5–2 times greater than that of VA and 1.3–2 times greater than that of *p*-CA. For mixtures, the uptake of FA was 3.0–4.6 times greater than that of VA and 2.9–8.0 times greater than that of *p*-CA.

The following results do not include the three-way mixtures because their

TABLE 1. UPTAKE OF FERULIC (FA), VANILLIC (VA) AND *p*-COUMARIC ACID (*p*-CA) BY CUCUMBER SEEDLINGS FROM 100 ML OF TREATMENT SOLUTION AFTER 8 HOURS

Treatment concentration (mM)	Uptake (μmol/g RFW)[a]		
	FA	VA	pCA
0.125	12.25 (0.67)[b]		
0.250	14.42 (0.84)		
0.500	18.32 (0.57)		
1.000	24.80 (1.24)		
0.125		5.81 (0.25)	
0.250		7.62 (0.48)	
0.500		11.43 (1.26)	
1.000		17.06 (1.04)	
0.125			5.94 (0.69)
0.250			10.75 (0.09)
0.500			11.03 (0.59)
1.000			16.90 (0.87)
0.125 + 0.125	9.62 (0.30)	2.53 (0.28)	
0.250 + 0.250	14.01 (0.06)	3.70 (0.02)	
0.500 + 0.500	17.92 (1.40)	3.88 (0.23)	
0.125 + 0.125	8.28 (0.60)		1.96 (0.31)
0.250 + 0.250	11.61 (1.34)		2.10 (0.07)
0.500 + 0.500	15.35 (1.32)		1.93 (0.22)
0.125 + 0.125		5.11 (0.09)	5.53 (0.16)
0.250 + 0.250		6.07 (0.42)	6.06 (0.32)
0.500 + 0.500		7.56 (0.40)	5.88 (0.23)
0.083 + 0.083 + 0.083	6.87 (0.23)	2.32 (0.11)	2.37 (0.11)
0.167 + 0.167 + 0.167	10.62 (0.62)	2.87 (0.24)	3.01 (0.34)
0.333 + 0.333 + 0.333	14.25 (1.17)	3.66 (0.51)	2.83 (0.22)

[a] RFW = root fresh weight.
[b] Standard error ($N = 3$).

individual phenolic acid concentrations were different from those in the rest of the treatments. The uptake for single phenolic acid treatments at the appropriate concentration was used as the control level. Only significant differences ($P < 0.05$) are presented. The uptake of VA and *p*-CA by cucumber seedlings was reduced in the presence of FA (Table 1). Uptake by seedlings was reduced 52–66% for VA and 67–82% for *p*-CA. For the VA and *p*-CA mixture, uptake of VA and *p*-CA by seedlings was reduced 34% (0.5 + 0.5 mM treatment) and 44–65% (0.25 + 0.25 and 0.5 + 0.5 mM treatments), respectively. Uptake of FA by seedlings in the presence of *p*-CA was reduced 32% (0.125 + 0.125 mM treatment). Uptake of FA in the presence of VA was reduced 22% (0.125 + 0.125 mM treatment).

Phosphorus Uptake. A summary of the P uptake data used in the joint action analysis is presented in Table 2. All three phenolic acids inhibited the uptake of P for 15-day-old cucumber seedlings, i.e., the slope (β_3) was significantly different from zero; Tables 3 and 4. Inhibition of P uptake generally increased as the phenolic acid treatment concentrations increased (Table 2).

TABLE 2. UPTAKE OF PHOSPHORUS (P) BY CUCUMBER SEEDLINGS FROM 100 ML OF TREATMENT SOLUTIONS AFTER 8 HOURS

Treatment concentration (mM)	P uptake (mg P/g RFW)[a]	Treatment concentration (mM)	P uptake (mg P/g RFW)
Control	0.40 (0.03)[b]	FA + VA	
		0.125 + 0.125	0.32 (0.03)
FA[c]		0.250 + 0.250	0.34 (0.03)
0.125	0.35 (0.03)	0.500 + 0.500	0.28 (0.02)
0.250	0.33 (0.03)		
0.500	0.30 (0.02)	FA + pCA	
1.000	0.27 (0.04)	0.125 + 0.125	0.33 (0.01)
		0.250 + 0.250	0.34 (0.01)
VA		0.500 + 0.500	0.24 (0.03)
0.125	0.40 (0.05)		
0.250	0.39 (0.04)	VA + pCA	
0.500	0.31 (0.02)	0.125 + 0.125	0.35 (0.03)
1.000	0.34 (0.01)	0.250 + 0.250	0.37 (0.05)
		0.500 + 0.500	0.33 (0.04)
p-CA			
0.125	0.39 (0.06)	FA + VA + pCA	
0.250	0.34 (0.02)	0.083 + 0.083 + 0.083	0.36 (0.02)
0.500	0.29 (0.01)	0.167 + 0.167 + 0.167	0.35 (0.05)
1.000	0.29 (0.04)	0.333 + 0.333 + 0.333	0.31 (0.02)

[a] Milligrams of phosphorus per gram root fresh weight.
[b] Standard error ($N = 3$).
[c] FA = ferulic acid, VA = vanillic acid, *p*-CA = *p*-coumaric acid.

TABLE 3. SUMMARY OF JOINT-ACTION ANALYSIS OF EFFECTS OF FERULIC ACID (FA), VANILLIC ACID (VA) AND *p*-COUMARIC ACID (*p*-CA) ON PHOSPHORUS UPTAKE OF 15-DAY-OLD CUCUMBER SEEDLINGS BASED ON INITIAL PHENOLIC ACID TREATMENT CONCENTRATION

			FA	VA	pCA
β_1^a (control value, mg P/g RFW)[b]	0.40				
β_2 (ln dose required for 50% inhibition)	0.82				
Values in mM			2.27	6.73	3.00
β_3 (rate parameter)	0.71[c]				
β_{4j} (relative potency)			1.00	0.34[d]	0.76
β_{5j} (coefficient of synergy; 0: similar or independent joint action, +: synergistic, −: antagonistic)		FA		−0.05	−0.1
		VA			−0.60[c]

[a] See data analysis section for details.
[b] Milligrams of phosphorus per gram root fresh weight.
[c] Significantly different from 0 at the 5% level.
[d] Significantly different from 1 at the 5% level.

TABLE 4. SUMMARY OF JOINT-ACTION ANALYSIS OF EFFECTS OF FERULIC ACID (FA), VANILLIC ACID (VA) AND *p*-COUMARIC ACID (*p*-CA) ON PHOSPHORUS UPTAKE OF 15-DAY-OLD CUCUMBER SEEDLINGS BASED ON PHENOLIC ACID UPTAKE

			FA	VA	pCA
β_1^a (control value, mg P/g RFW)[b]	0.40				
β_2 (ln dose required for 50% inhibition)	3.71				
Values in μmol/g RWF			40.76	50.02	34.10
β_3 (rate parameter)	1.29[c]				
β_4 (relative potency)			1.00	0.81	1.20
β_5 (coefficient of synergy; 0: similar or independent joint action, +: synergistic, -: antagonistic)		FA		−0.24	0.10
		VA			−0.30

[a] See data analysis section for details.
[b] Milligrams of phosphorus per gram root fresh weight.
[c] Significantly different from 0 at the 5% level.

Effects of various equimolar mixtures of phenolic acids (with the exception of the VA and *p*-CA mixture; Table 3) on the uptake of P when compared to the effects of individual phenolic acids were found to be additive, i.e., the coefficients of synergy (β_5) were not significantly different from zero; Tables 3 and 4. The effects of VA and *p*-CA mixtures (Table 3) on P uptake, when compared to the effects of individual phenolic acids, were antagonistic.

The dose (mM) for 50% inhibition of P uptake based on the initial treatment concentrations was significantly higher (2.2–3.0 times) for VA than for *p*-CA and FA (Table 3). This was not the case when dose for 50% inhibition was based on phenolic acid uptake (Table 4). The 50% doses for FA and *p*-CA were not significantly different, i.e., relative potencies (β_4) were not significantly different; Tables 3 and 4.

DISCUSSION

The effects of phenolic acids on P uptake of cucumber seedlings clearly are determined by concentration, or more specifically, by the number of molecules interacting with root membranes (Glass, 1974b, 1975). As a way of estimating phenolic acid–root interactions, we determined uptake of the phenolic acids by monitoring solution depletion. Shann and Blum (1987) noted for cucumber seedlings that, although uptake based on isotopes more accurately reflected uptake of phenolic acids, depletion from solution was useful in describing trends over short time periods. Similar observations were made for the uptake of phenolic acids by water hyacinth (Keeffe et al., 1987). Shann and Blum (1987) also reported interference in the uptake of one phenolic acid by another phenolic acid. Such interference was clearly evident for the three phenolic acids under study.

In these experiments, uptake of phenolic acids and P was determined 8 hr after treatment. This time period was chosen because the effects of phenolic acids on P uptake from the 100 ml treatment solutions were not clearly evident after 4 hr. Given the 8-hr period, we suspect that uptake of phenolic acids was overestimated, since microbial and physicochemical breakdown of those acids would be included. Microbial action on phenolic acids, however, was nutrient limited in the KH_2PO_4–$CaSO_4$ system. Uptake should be more meaningful than the initial treatment concentrations in determining plant response to phenolic acids, since uptake should more closely reflect root–phenolic acid interactions. The effective dose (i.e., the amount of a compound that actually reaches its target) required for 50% inhibition of P uptake based on the initial treatment concentrations could thus be substantially over- or underestimated because of differences in root–phenolic acid interactions (i.e., membrane solubility; Glass, 1974b, 1975) and activity (toxicity) of individual phenolic acids or mixtures of phenolic acids.

The relative potency (β_4) of VA based on initial treatment concentrations (ITC) was significantly different from (lower than) 1 (Table 3). The 50% VA-inhibition dose for P uptake was more than two times higher than FA. The relative potency of VA based on phenolic acid uptake (PAU) was not, however, significantly different from FA (Table 4). These observations together suggested that the root–VA interactions were much lower than the root–FA interactions. This conclusion is consistent with the phenolic acid uptake data (Table 1). Uptake of VA was 1.5–4.6 times lower than that for FA.

Relative potencies of both joint-action analyses (ITC and PAU; Tables 3 and 4) suggested that *p*-CA was as active as FA in inhibiting P uptake. This observation, however, is in conflict with the phenolic acid uptake data (Table 1). Uptake of FA was up to eight times higher than *p*-CA. This suggested that *p*-CA must be much more inhibitory to P uptake than FA. One possible resolution for this conflict is that the greater activity (toxicity) of *p*-CA was balanced by the reduction in the root–*p*-CA interactions (i.e., lower uptake).

The coefficient of synergy (β_5) based on ITC for *p*-CA and VA was negative and significantly different from zero (Table 3). Thus the effects of mixtures of *p*-CA and VA on P uptake, when compared to the effects of the individual phenolic acids on P uptake, were antagonistic. The joint action of two compounds is antagonistic if, when applied together, one decreases the effectiveness of the other. The coefficient of synergy for *p*-CA and VA based on PAU, however, was not significantly different from zero (Table 4). The latter observation suggested that the antagonistic effects noted for *p*-CA and VA were due to lower root–phenolic interactions of *p*-CA or VA in mixtures. The uptake data lend support to this conclusion (Table 1). For example, *p*-CA and VA uptake was reduced 34 and 65%, respectively from the 0.5 mM mixture solution when compared to the uptake from the individual phenolic acid solutions.

The joint action of these three phenolic acids on P uptake were, with one exception (i.e., *p*-CA and VA based on ITC), additive. In other words, the effects on P uptake by incremental increases in concentration of one compound were not dependent on the presence of another compound. The data further suggest that as the number of phenolic acids increased in a mixture, the concentrations of the individual phenolic acids in that mixture required to bring about a given response declined. This has been shown also for cucumber leaf expansion (Blum et al., 1989).

Finally, proper interpretation of the role of individual phenolic acids in mixtures on P uptake requires an understanding of not only plant response based on joint action analysis, but also the activity (i.e., toxicity) and levels of root–phenolic acid interactions. Data on uptake of phenolic acids in conjunction with joint action analysis of P uptake can be helpful in identifying potential actions of individual phenolic acids in mixtures. In addition, the results suggest that low concentrations of individual phenolic acids in the complex mixtures

observed in field soils may be important in determining essential nutrient uptake by sensitive species.

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PREFERENCE OF MEADOW VOLES (*Microtus pennsylvanicus*) FOR CONIFER SEEDLINGS: CHEMICAL COMPONENTS AND NUTRITIONAL QUALITY OF BARK OF DAMAGED AND UNDAMAGED TREES

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Abstract—Meadow voles (*Microtus pennsylvanicus* Ord.) of the 1987–1988 population density peak left several seedling plantations of Norway spruce (*Picea abies*) and Norway pine (*Pinus resinosa*) severely damaged in southern Quebec, Canada, while white spruce (*Picea glauca*) and white pine (*Pinus strobus*) suffered no damage. We compared levels of crude protein, total nonstructural carbohydrates (TNC), total phenols, and monoterpenes in the bark to detect differences between damaged and undamaged seedlings within and between plantations. There were no significant differences in levels of nutritional components between both types of seedlings within the same plantation. However, differences were noted among tree plantations for protein, TNC, and phenolics content but these differences could not be assigned to the voles' selection for particular species. Quantitative and qualitative differences also were registered in monoterpene levels between species. β-Myrcene and bornyl acetate were found only in seedlings of undamaged species. Moreover, the latter contained higher levels of limonene. We conclude that the presence of specific monoterpenes is more important in defense mechanisms of conifer seedlings than relative levels of nutrients (protein or TNC) or total phenolics.

Key Words—*Picea*, *Pinus*, meadow voles, *Microtus pennsylvanicus*, selection, monoterpenes, phenolics, protein, nonstructural carbohydrates, plant defense.

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INTRODUCTION

The meadow vole (*Microtus pennsylvanicus* Ord.) is regarded as one of the major mammalian pests in orchards and tree plantations in North America (Cayford and Haig, 1961; Radvanyi, 1974). It can inflict extensive damage during winter months of peak years when snow cover is well established (Buckner, 1972). For unknown reasons some plantations are severely damaged while others are free of any vole attack. It was suggested that this patchy distribution of damage level could be related to spatial variation in vole densities caused by spatial heterogeneity in suitable microhabitats (Von Althen, 1979; Byers et al., 1976). On the other hand, it is known that certain conifer species are more susceptible to vole attack than others (Littlefield et al., 1946; Cayford and Haig, 1961; Sartz, 1970; Buckner, 1972).

Preference of herbivores for conifers has been associated with the presence of defense compounds such as terpenes (Santamour et al., 1963; Farentinos et al., 1981; Sinclair et al., 1988) or total phenols (Rousi and Häggman, 1984). However, Hansson et al. (1986) did not find any clear relationship between levels of phenolic or terpenic substances in trees and debarking damage indices established after population peaks of voles. These studies did not simultaneously measure both the positive (protein, nonstructural carbohydrates) and negative (phenols, terpenes) nutritional components that herbivores ingest in bark tissue. All have been associated with selection of voles for trees (Sullivan and Sullivan, 1982; Green, 1978; Rousi and Häggman, 1984). High protein content in diets can reduce or eliminate the negative effects of phenolic compounds (Lindroth and Batzli, 1984). This explains why voles show strong preferences for herbaceous species with high protein/total phenols ratios (Bergeron and Jodoin, 1987; Bucyanayandi and Bergeron, 1990).

In this paper, we report comparisons of levels of protein, total nonstructural carbohydrates, total phenols, and monoterpenes between bark samples of seedlings sampled in plantations of Norway spruce (*Picea abies*), white spruce (*Picea glauca*), Norway pine (*Pinus resinosa*), and white pine (*Pinus strobus*) to relate voles' debarking to nutritional quality of bark. We hypothesize that the bark of damaged seedlings contains more protein and nonstructural carbohydrates and less phenolic compounds and/or monoterpenes than undamaged seedlings and so are high in nutritional quality to voles.

METHODS AND MATERIALS

In the early spring of 1988, after snow melt, we visited 18 pure stands of conifer species (six Norway spruce, five Norway pine, four white spruce, and three white pine) in southern Quebec within a radius of 60 km around Sher-

brooke University. In this region, voles had reached their peak densities in the summer of 1987 and the winter of 1988. Our observations showed that heavy debarking occurred in stands of Norway spruce and Norway pine, while plantations of white spruce and white pine suffered no damage. We were confident that this type of damage was caused by voles because it was similar to that observed in experiments being made within enclosures with the same species where only voles were present. Injured trees of damaged species were then randomly sampled at a rate of 8–10 trees from two different plantations along with their nearest undamaged neighbor. Ten trees were also selected in two undamaged plantations each of white spruce and white pine.

All plantations sampled showed comparable signs of vole activity (runways apparent), although this parameter was not quantified. All plantations were 4 years old, except those of Norway spruce, which were 7 years old. This difference in plant maturity would not be important in our study because voles are known to attack trees in plantations up to 15 years of age (Hansson, 1985). Seedlings were cut and brought to the laboratory in plastic bags. The bark accessible to voles (0–30 cm above the ground level) was then rapidly removed and cut into fragments of about 1 cm. For determination of total nonstructural carbohydrates (TNC), a subsample was oven-dried at 100°C for 90 min and then at 70°C to constant mass (Smith, 1969). Another subsample was oven-dried at 60°C for 48 hr for protein and total phenolics analyses. Because phenols could complex with protein at high temperature, Julkunen-Tiitto (1985) proposed that samples should be dried at less than 50°C to get more reliable results. However, we did not find differences in total phenolics levels between samples dried at 45°C and 60°C (Bucyanayandi and Bergeron, unpublished data). The dried material was ground to 0.5 mm diameter in a Brinkmann mill and then frozen at −20°C until analysis, which was completed within four months of sampling. The remainder of bark sample was immediately frozen for monoterpene determination.

Total nonstructural carbohydrates (TNCs) were measured according to the procedures of daSilveira et al. (1978) using amyloglucosidase (Sigma Chemical Co.) for digestion. After the enzyme digestion, hydrolysis of carbohydrates to monomers was completed with 0.2 N sulfuric acid (Smith, 1969). Nitrogen (N) content was determined by the micro-Kjeldahl method developed by Lang (1958). Crude protein was then obtained by multiplying N (%) by 6.25. Total phenols were determined following the method of Singleton and Rossi (1965) using the Folin-Ciocalteu reagent (BDH Chemical Co.). Extractions were made according to the procedures of Gartlan et al. (1980). All analyses were run in duplicate samples, and results were expressed as percent of dry mass.

For monoterpene analysis, 3 g of fresh bark samples were crushed to a powder in a mortar with liquid nitrogen and extracted with 40 ml of pentane

for 2 hr using a Soxhlet extractor. The pentane extract then was transferred to a 50-ml volumetric flask to increase the volume to 50 ml. The monoterpene composition was analyzed by gas chromatography (Hewlett & Packard, model 5890) equipped with a flame-ionization detector, a 15-m × 0.3-mm cross-linked 5% phenylmethyl silicone column, and an integrator. A 2-μl sample was injected into a glass-lined injection port at a split ratio of 100:1. The oven conditions were 2 min at 50°C, then increased by 5°C/min up to 150°C to be left at this isothermal temperature for 4 min. The carrier gas was helium, supplied at 1 ml/min. The monoterpenes were identified by comparing retention times of unknowns in bark samples with those of standard commercial compounds and by GC-MS. Because we operated under constant conditions, the concentration of a component was proportional to its peak area. We then used this parameter (corrected for response factor) as a measure of terpenes content in statistical analyses (Hanover, 1966).

Comparisons between damaged and undamaged trees within each conifer plantation were performed using the Wilcoxon two-sample test. One-way analysis of variance on ranks permitted an evaluation of differences in levels of chemical components among conifer species. All statistical analyses were performed on the computing facilities of Sherbrooke University using SAS programs (SAS, 1985).

RESULTS

Four of the six stands of Norway spruce and three of the five Norway pine stands suffered seedling losses. Plantations of white spruce and white pine did not show any sign of debarking, leading us to think that these species were less palatable to voles and must possess specific compounds for defense against herbivores.

Protein, TNC, Phenolics. Our survey yielded two plantations each of Norway spruce and Norway pine that had been damaged by voles and two plantations each of white spruce and white pine left untouched. Phenolic levels in Norway spruce averaged 6%, while protein content varied from 6.6% to 7.6%, with no significant differences between damaged and undamaged seedlings within the same plantation (Figure 1). Bark samples of Norway pine contained 4.8–5.4% phenols and 5.5–7.7% protein. No significant differences were detected between damaged and undamaged stems. Variability in TNC content was very large, with values ranging from 8% (Norway pine) to 28% (Norway spruce) (Figure 1). Interspecific comparisons showed that bark of white pine contained significantly lower levels of phenols and higher content of protein and TNC (Table 1). In contrast, white spruce was higher in phenols and relatively lower in protein. Milton (1979) reported that digestion-inhibition ratios

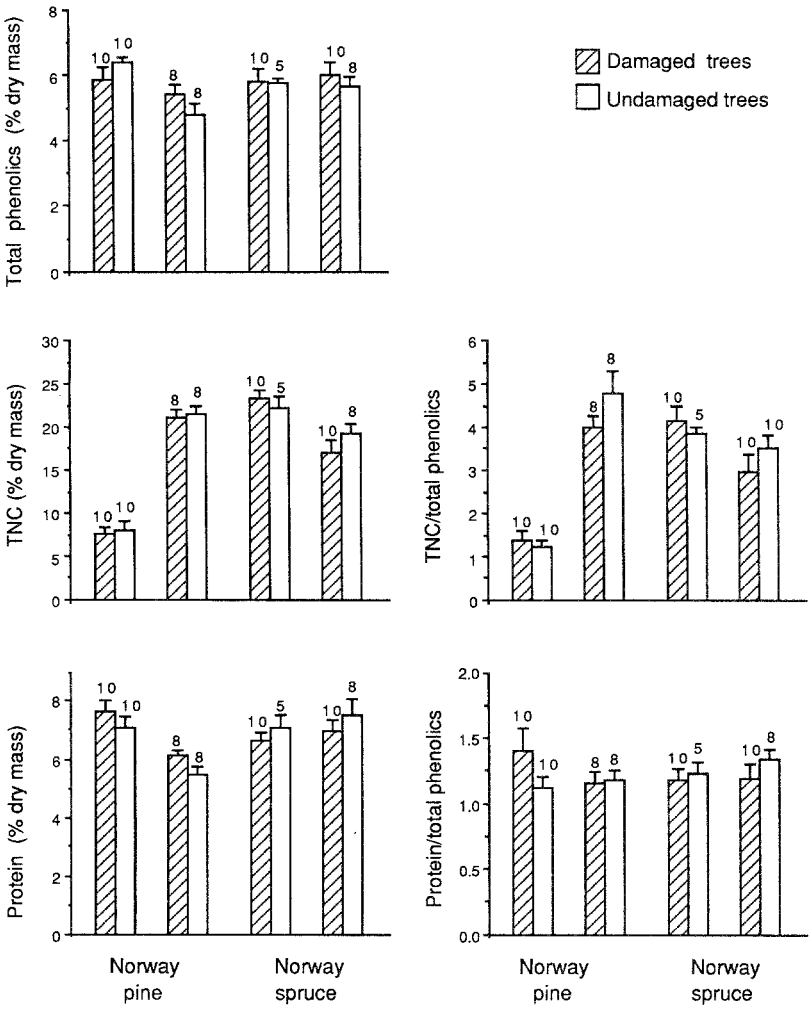


FIG. 1. Levels of total phenolics, total nonstructural carbohydrates (TNC), crude protein, TNC–total phenolics ratios, and protein/total phenolics ratios between damaged and undamaged seedlings from two different plantations (left and right pair of bars) of Norway pine and Norway spruce. Sample size is indicated above each standard error bar. All values are expressed as percentage unit dry mass.

involving positive nutritive factors as numerators (protein, TNC) and negative ones as denominators (phenols) were better estimators of food quality than compounds taken individually in herbivory studies. This revealed that protein–phenolic ratios were significantly higher in white pine, lower in white spruce,

TABLE 1. LEVELS OF SELECTED NUTRITIONAL COMPONENTS IN BARK SAMPLES OF SEEDLINGS FROM DAMAGED AND UNDAMAGED CONIFER PLANTATIONS[a]

Conifer species	N	Dry mass (%)			Protein/phenols	TNC/ Phenols
		Protein	TNC	Phenols		
Damaged						
Picea abies 1	15	6.79bc	22.98d	5.81c	1.20b	4.05bc
		(0.24)	(0.79)	(0.24)	(0.06)	(0.21)
Picea abies 2	18	7.23abc	18.05e	5.89b	1.26b	3.21d
		(0.33)	(1.01)	(0.24)	(0.07)	(0.26)
Pinus resinosa 3	20	7.37ab	7.76f	6.13b	1.26b	1.30e
		(0.27)	(0.63)	(0.23)	(0.09)	(0.12)
Pinus resinosa 4	20	5.82d	21.33d	5.11c	1.17b	4.37b
		(0.16)	(0.64)	(0.23)	(0.05)	(0.31)
Undamaged						
Picea glauca 5	10	6.39cd	24.34bc	7.18a	0.92c	3.49cd
		(0.49)	(1.33)	(0.32)	(0.10)	(0.28)
Picea glauca 6	10	7.57ab	24.01bc	8.01a	0.97c	3.05d
		(0.32)	(0.80)	(0.33)	(0.07)	(0.19)
Pinus strobus 7	10	8.00a	25.37b	2.13d	3.81a	12.23a
		(0.42)	(0.53)	(0.11)	(0.20)	(0.73)
Pinus strobus 8	10	7.46ab	28.59a	1.97d	3.80a	14.55a
		(0.36)	(0.81)	(0.05)	(0.20)	(0.39)
Mean squares		4376.42	10648.67	10147.37	7944.11	11244.01
F		5.89	38.28	29.90	16.09	47.63
$P > F$		0.0001	0.0001	0.0001	0.0001	0.0001
df		7	7	7	7	7

[a] Values represent mean, with standard errors in parentheses. Different letters within a column show significant differences (one-way analysis of variance on ranks followed by the Duncan's multiple-range test). TNC = total nonstructural carbohydrates. Number allocated to each conifer species refers to a different plantation.

and intermediate in Norway spruce and Norway pine. TNC–phenolic ratios were also significantly higher in white pine than in other species.

Monoterpenes. Four monoterpenes, α-pinene, β-pinene, 3-carene, and limonene (possibly with β-phellandrene since the column used could not separate the two compounds), were found in bark samples of pines and spruces. β-Myrcene and small quantities of bornyl acetate were detected only in white pine and white spruce, two species that had not suffered any damage by voles. Furthermore, camphene and borneol were found only in white pine. None of the latter four compounds could be found in Norway spruce and Norway pine that suffered vole debarking. There were no significant differences in terpene levels between damaged and undamaged seedlings (Norway spruce and Norway pine) within the same plantation or between different plantations for the same

TABLE 2. DIFFERENCES IN MAJOR MONOTERPENES FOUND IN BARK OF DAMAGED AND UNDAMAGED CONIFER SPECIES[a]

	Conifer species					
	Damaged		Undamaged			
Monoterpene	Norway spruce	Norway pine	White pine	White spruce	F	$P > F$
α-Pinene	14.22c (23)	52.70a (23)	42.83b (12)	36.00b (12)	36.70	0.0001
β-Pinene	19.52c (23)	38.70b (23)	41.33b (12)	54.17a (12)	13.16	0.0001
3-Carene	23.04b (23)	50.26a (23)	26.78b (9)	25.82b (11)	14.92	0.0001
Limonene	32.35b (23)	17.74c (23)	51.92a (12)	59.17a (12)	34.45	0.0001
Bornyl acetate			15.83a (12)	8.09b (11)	9.88	0.0049
β-Myrcene			9.25b (12)	15.00a (11)	4.85	0.0390
Camphene			6.00 (11)			
Borneol			6.50 (12)			

[a] Values represent mean rank, with the sample size in parentheses. Different letters within a row indicate significant differences (one-way analysis of variance on ranks followed by the Duncan's multiple-range test).

species. This permitted us to pool data sets for single species and make interspecific comparisons. There were significant differences in levels of all monoterpenes between the four conifer species, but only limonene differentiated undamaged from damaged species (Table 2). Content of β-pinene was higher in white spruce, which is a damage-free species, while α-pinene and 3-carene were higher in Norway pine, which is known to be often damaged by voles in peak years.

DISCUSSION

All conifer species had bark protein levels that can barely satisfy the minimum requirements for voles when they have to detoxify phenolic compounds (Lindroth and Batzli, 1984). Moreover, doses of phenols were within the range that makes food resources unpalatable or less attractive for voles (Bergeron and Jodoin, 1987) because they complex with digestive enzymes and protein of

plants. This in turn decreases digestibility and assimilation rates of plants (Rhoades and Cates, 1976). Seedlings thus can be regarded merely as subsistence food items that voles ingest in winter.

Although our survey revealed that some conifer plantations could escape one population peak of meadow voles, data presented here do not permit us to ascertain whether or not these plantations had been protected by defense compounds in seedlings or by the absence of high population densities at specific locations. Bark of white spruce contained the highest levels of phenolics among the four species tested and the lowest digestion-inhibition ratios, which would make them the least palatable species. However, white pine showed low levels of phenolics and higher content of protein and carbohydrates, making it a supposedly highly palatable candidate for herbivores. In spite of this, plantations of white pine were free of any vole damage. Bergeron and Jodoin (1989) found that damaged plantations showed significant differences in plant biomass patterns in terms of preferred food items of meadow voles, inferring that voles do have habitat preferences and might not be able to reach dense populations in all plantations because of a lack of suitable food resources. However, field studies in enclosures confirmed the selection of voles for Norway spruce and Norway pine (Roy and Bergeron, 1990; Bucyanayandi and Bergeron, in preparation).

Phenolic compounds that have been negatively related to food selectivity of voles for herbaceous species (Bergeron and Jodoin, 1987; Marquis and Batzli, 1989) do not seem to play an active role in defense of conifer seedlings. In addition, our results do not support the idea that voles eat the bark of trunks and lateral branches in winter because of their high content in TNC (Hansson and Boström in Rousi, 1983; Green, 1978). We have found that species with low carbohydrate content, such as the red pine, could be damaged by voles while another, such as the white spruce, in spite of significantly higher levels of TNC, was left undamaged.

Two of the three monoterpenes (α-pinene, β-pinene, and 3-carene) that could not be associated in this study to the selection of voles for conifer seedlings have been found before to play an important role against pest species in other studies. Farentinos et al. (1981) showed that squirrels (*Sciurus aberti*) fed on α-pinene-poor twigs of *Pinus ponderosa* during winter. However, Fenton (1962) found a positive correlation between levels of α-pinene in *Pinus virginiana* and damage rate by wild mice. One of the recent studies of Cates et al. (1987) indicated that high dosages of β-pinene in experimental diets increased growth and survivorship of western spruce budworm (*Choristoneura occidentalis*). These conflicting results suggest that different coevolutionary processes are at work in different herbivorous species.

Although biological effects of monoterpenes specific to undamaged species in our study are not known for higher vertebrates, they have been studied in insects. For example, limonene and myrcene have been shown to be very toxic

to the southern pine beetle (*Dendroctonus frontalis*) while camphene was the least toxic (Coyne and Lott, 1976). Growth and survival of spruce budworm larvae have been reduced by bornyl acetate added to laboratory diets (Cates et al., 1987). This suggests that high levels of myrcene, limonene, bornyl acetate, and possibly borneol may be involved in defense of conifers against vole debarking. Further work on feeding behavior of voles using varying dosages of the latter compounds should be initiated to determine if a particular compound or a mixture of terpenes is needed in this defense. Nutrition of herbivores also is related to minerals and fibers (Goldberg et al., 1980; Rodgers and Lewis, 1985; Chapin et al., 1986), two features that were not studied in this report. Most of these components are tied to taste, and one wonders if herbivores can really discriminate that much to establish their food selection patterns.

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STRUCTURE OF A NOVEL PHYTOECDYSTEROID, VITEXIRONE, AND EFFICIENT ISOLATION OF PHYTOECDYSTEROIDS FROM *Vitex fisherii*

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Abstract—A novel phytoecdysteroid, vitexirone, has been isolated from a MeOH extract of the root bark of the East African medicinal plant *Vitex fisherii* by recycling high-performance liquid chromatography on a semipreparative scale. In addition, three known phytoecdysteroids, 20-hydroxyecdysone, ajugasterone C, and turkesterone, also were isolated. The structure of vitexirone has been established spectroscopically. The position and stereochemistry of the 11-α-hydroxy group of ajugasterone C and vitexirone were confirmed by the ^1H–^1H homonuclear COSY NMR data. These phytoecdysteroids disrupt the molting process of the pink bollworm *Pectinophora gossypiella*.

Key Words—*Vitex fisherii*, Verbenaceae, phytoecdysteroid, vitexirone, 20-hydroxyecdysone, ajugasterone C, turkesterone, pink bollworm, Pectinophora gossypiella, Lepidoptera, Gelechiidae.

INTRODUCTION

In the course of our continuing studies on hormonal interactions between plants and insects, we have identified many phytoecdysteroids in tropical medicinal plants (Kubo et al., 1981, 1984a; Kubo and Klocke, 1983). These phytoecdysteroids disrupt normal development of several insect pests in feeding assays with an artificial diet (Kubo et al., 1983, 1984b). This report deals with an efficient isolation and identification of four phytoecdysteroids from a MeOH extract of the root bark of the East African medicinal plant, *Vitex fisherii* (Verbenaceae).

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METHODS AND MATERIALS

Infrared and Ultraviolet Spectrometry. Infrared spectra were acquired on a Perkin-Elmer model 1310 IR in KBr. Ultraviolet spectra were acquired on a Hitachi model 100-80 spectrophotometer in MeOH.

Proton and Carbon-13 Magnetic Resonance. The spectra were acquired in C_5D_5N on either a Brucker model 400 (400 MHz for [^{1}H]NMR, 100 MHz for [^{13}C]NMR) or a JEOL model GX-400 (400 MHz for [^{1}H]NMR, 100 MHz for [^{13}C]NMR) spectrometer. The chemical shifts were assigned relative to C_5H_5N or TMS.

Mass Spectrometry. The EIMS spectra were acquired on a JEOL model DX303-HF double-focusing mass spectrometer equipped with a JMA-DA5000 data system. The instrument was used in the electron ionization mode at 70 eV with an ion source temperature of 250°C.

Droplet Counter Current Chromatography. DCCC was performed on a model DCC-G2 (Tokyo Rikakikai Co., Tokyo, Japan). The solvent system used was $CHCl_3$–MeOH–H_2O (13:7:4, v/v/v). The upper phase was used as the mobile phase to elute the components. The chromatography was monitored by gravimetric measurement of each fraction (1.5 ml).

Recycling High-Performance Liquid Chromatography. R-HPLC was performed on a model JAI LC-09 (Japan Analytical Industry Co., Tokyo, Japan). The column used on the R-HPLC was an Asahipack GS-320 (50 cm × 2.5 cm, 5 μm), which is packed with polyvinyl alcohol resin and which depends principally on molecular size for separation. HPLC-grade MeOH was used as the eluant at a flow rate 3 ml/min. The detection method used was UV at λ 254 nm.

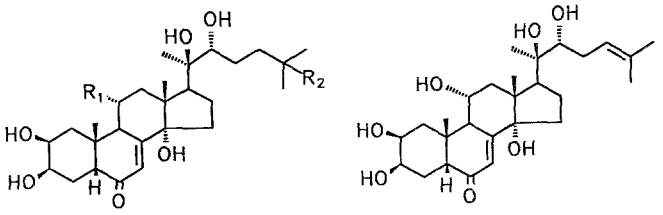
Plant Material. The root bark (500 g) of *V. fisherii* was collected near Kakamega, Kenya, and air dried. The dried root bark was ground and covered with MeOH. The MeOH solution was stored at ambient temperature.

Extraction and Isolation. Following extraction with MeOH, the MeOH solution was condensed by evaporation. The 1 g of the MeOH extract was separated using DCCC and R-HPLC. An artificial diet feeding assay using the pink bollworm, *P. gossypiella*, was used as a guide during the isolation procedure.

Bioassay. The artificial diet feeding test against the insect followed the literature (Kubo and Klocke, 1983).

RESULTS

Isolation. The isolation of three known phytoecdysteroids, turkesterone **1** (Usmanov et al., 1975), 20-hydroxyecdysone **2** (Takemoto et al., 1967), and ajugasterone C **3** (Imai et al., 1969) as well as the novel vitexirone **4** (Scheme 1), was achieved using DCCC (Kubo and Hanke, 1987) followed by R-HPLC.



1 $R_1 = R_2 = OH$
2 $R_1 = H$, $R_2 = OH$
3 $R_1 = OH$, $R_2 = H$

4

SCHEME 1.

DCCC yielded compounds **1** and **2** in 4.4% and 18% yield, respectively, from the MeOH extract and a mixture including compounds **3** and **4** (Figure 1). The R-HPLC gave compound **3** in 11% yield and slightly impure compound **4** in ca 2.5% yield from the MeOH extract (Figure 2).

The R-HPLC technique was especially useful in the final purification of these rather polar and structurally related phytoecdysteroids, since the chemical properties of the four compounds were very similar. As shown in Figure 2, the single peak in the first chromatogram was completely separated into three peaks after recycling five times on the R-HPLC. Two of the peaks were compounds **3** and **4.** The third peak was biologically inactive.

Structure Determination. The UV, IR, and NMR spectra of compounds **1–4** were all similar (Tables 1 and 2). The major compound **2** was identified as 20-hydroxyecdysone through spectroscopic comparison to an authentic sample.

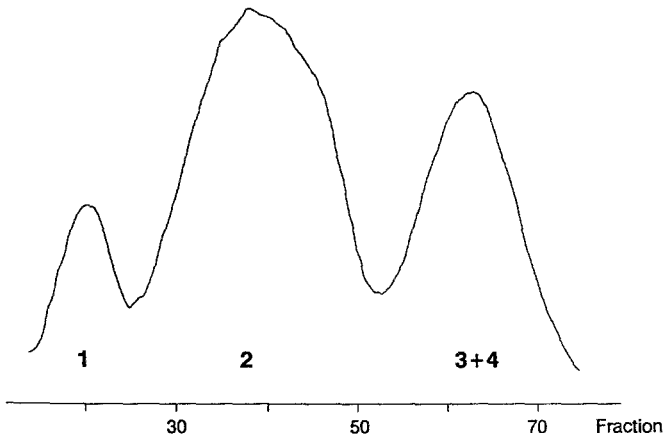


FIG. 1. DCCC of 1 g of a MeOH extract of *V. fisherii* root bark. Mobile phase: upper layer of $CHCl_3$–MeOH–H_2O (13:7:4, v/v/v). Detection: gravimetric, 1.5 ml/fraction. The mixture peak was separated on R-HPLC.

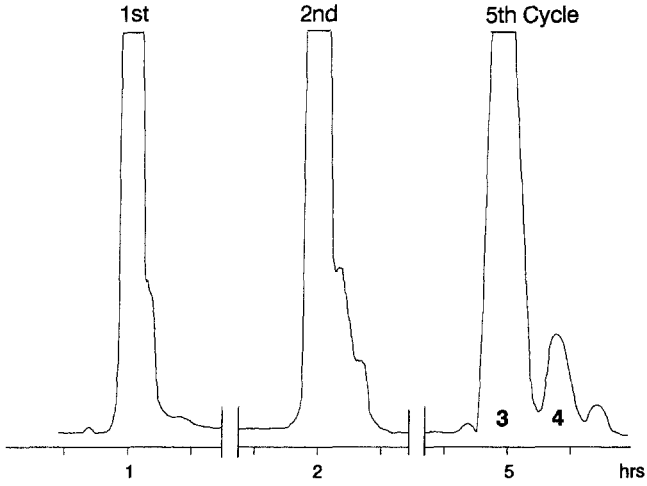


FIG. 2. Recycling HPLC chromatogram for the separation of **3** and **4**; column: Asahipack GS-320, 500 mm, × 25 mm, 5 μm; eluant: MeOH; flow rate: 3 ml/min; detection; UV λ 254 nm.

TABLE 1. [^{1}H]NMR CHEMICAL SHIFTS OF **1, 3,** AND **4** IN C_5D_5N

H	1	3	4
1α	3.46	3.44	3.44
2	4.60	4.60	4.60
3	4.22	4.21	4.22
5	3.10	3.11	3.03
7	6.32	6.30	6.29
9	3.62	3.87	3.88
11	4.60	4.60	4.60
12α	3.05	3.03	3.03
12β	2.83	2.70	2.71
18	1.33	1.33	1.33
19	1.28	1.28	1.26
21	1.59	1.58	1.59
22	3.62	3.79	3.88
24	5.54	5.53	5.53
26	1.37	0.83[a]	1.59
27	1.37	0.82[a]	1.66

[a] Interchangable.

TABLE 2. [^{13}C]NMR SPECTRAL DATA OF **1**, **3**, AND **4** IN C_5D_5N

C	1	2	3
1	39.9	39.5	39.9
2	68.1	68.1	68.2
3	68.4	68.4	68.9
4	32.9	32.9	32.9
5	52.5	52.5	52.5
6	203.0	203.8	203.6
7	122.0	122.3	122.3
8	164.0	164.1	164.2
9	42.8	42.8	42.8
10	39.5	39.9	39.5
11	68.8	68.9	68.9
12	44.2	44.2	44.1
13	48.2	48.2	48.2
14	84.2	84.2	84.2
15	32.0	31.9	31.9
16	21.5	21.5	21.5
17	50.0	50.0	50.0
18	19.0	19.0	19.0
19	24.9	24.9	24.9
20	76.8	76.8	76.9
21	21.6	21.5	21.5
22	77.5	76.7	76.7
23	27.5	30.3	31.8
24	42.6	37.1	123.7
25	69.5	28.2	131.9
26	30.0[a]	22.4[b]	18.0
27	30.1[a]	23.3[b]	25.9

[a,b]Interchangable.

The remaining compounds **1**, **3**, and **4** had an additional secondary hydroxy group in their steroidal skeleton as indicated by their [^{1}H]NMR signal at δ 4.6 ppm and [^{13}C]NMR signals at δ 68.8, δ 68.9, δ 68.9 ppm, respectively. This indicated that one of the methylene hydrogens in the steroid skeleton was replaced by a hydroxy group, as was established from the NMR data of compound **3**. In the ^{1}H–^{1}H homonuclear COSY (^{1}H–^{1}H COSY) spectrum of compound **3**, the signal at δ 6.30 ppm assigned to H-7 couples to the δ 3.87 ppm (H-9) signal, which has two coupling constants of J = 2.5 and 8.8 Hz. The smaller coupling constant of H-9 was assigned to the allylic coupling with H-7, and the larger coupling constant was assigned to the vicinal coupling with H-11 at δ 4.60 ppm. Therefore the extra hydroxy group should be located at C-11.

From the above data, compound **3** must be identical to ajugasterone C. However, the stereochemistry at C-11 remained unclear because the coupling constant of 8.8 Hz between H-11 and H-9 indicated that the dihedral angle between these protons deviated from 60°. In fact, a Dreiding model of compound **3** showed such deviation. Although it is clear that one of the geminal protons of H-12 at δ 2.70 ppm has two coupling constants of J = 5.7 and 11.7 Hz, the stereochemistry of the 11-hydroxy group could not be established from these coupling constants. Because the larger coupling constant must be assigned to the geminal coupling of the H-12 methylene and the smaller coupling constant could be assigned to the vicinal coupling between H-12 and H-11, the possibility of the α-equatorial stereochemistry of H-11 could not be eliminated. This ambiguity was only resolved by clarifying the coupling constants between H-11 and another H-12. To determine the coupling constant between H-11 and one of the H-12s, the slice method (Redfield and Boyd, 1986) in the ^{1}H–^{1}H COSY spectrum was applied (Figure 3).

The signals adjacent to C-11 were assigned first by the ^{1}H–^{1}H COSY spectrum of compound **3.** The δ 3.87 ppm (H-9) signal couples to the δ 4.60 ppm (H-11) signal, which couples to one of the δ 3.0 ppm (3H, m) protons and the δ 2.70 ppm proton. The signal corresponding to H-12, which overlapped with two other proton signals at δ 3.0 ppm, was extracted using the slice method in the ^{1}H–^{1}H COSY spectrum. The sliced and projected spectrum at δ 2.70 ppm (one of the H-12s) showed that the other H-12 at δ 3.0 ppm was a triplet having ca. 12 Hz coupling constant (Figure 3). To have two large coupling constants, H-12 at δ 3.0 ppm must be α-axial, because one coupling constant was assigned to the geminal coupling of the H-12 methylene and the other was assigned to the axial–axial coupling between H-11 and H-12 as the sole possibility. Thus H-11 was β-axial and the 11-hydroxy group must be α-equatorial. This assignment was supported by the fact that the chemical shift of H-12α at δ 3.0 ppm is lower than the shift of H-12β at δ 2.70 ppm. The deshielding effect for H-12α is caused by the 1,3-diaxial relation between H-12α and OH-14.

From the above observation, compound **1** was assigned as turkesterone. However, compound **4** was an unknown phytoecdysteroid.

Vitexirone. Compound **4,** vitexirone, gave the following spectroscopic data: EI-MS, *m/2* 461 ($M+1-H_2O$, $C_{27}H_{42}O_7+1-H_2O$); UV λ_{max} (MeOH) 234 nm; IR (KBr) 3400, 1655 cm^{-1}; [^{1}H]NMR (400 MHz, C_5D_5N), see Table 1; [^{13}C]NMR (100 MHz, C_5D_5N), see Table 2.

The presence of a $\Delta_{24\text{-}25}$ double bond was established by the existence of an additional olefinic proton at δ 5.53 ppm compared to ajugasterone C **3** and two vinyl methyl groups at δ 1.59 and δ 1.66 ppm instead of at δ 1.37 ppm for compound **3.** The stereochemistry of the 11-hydroxy group in **4** was identical to that of ajugasterone C **3** from the [^{1}H]NMR.

Growth Inhibition. All four isolated phytoecdysteroids cause growth inhibition in larvae of the pink bollworm, *Pectinophora gossypiella*, as well as

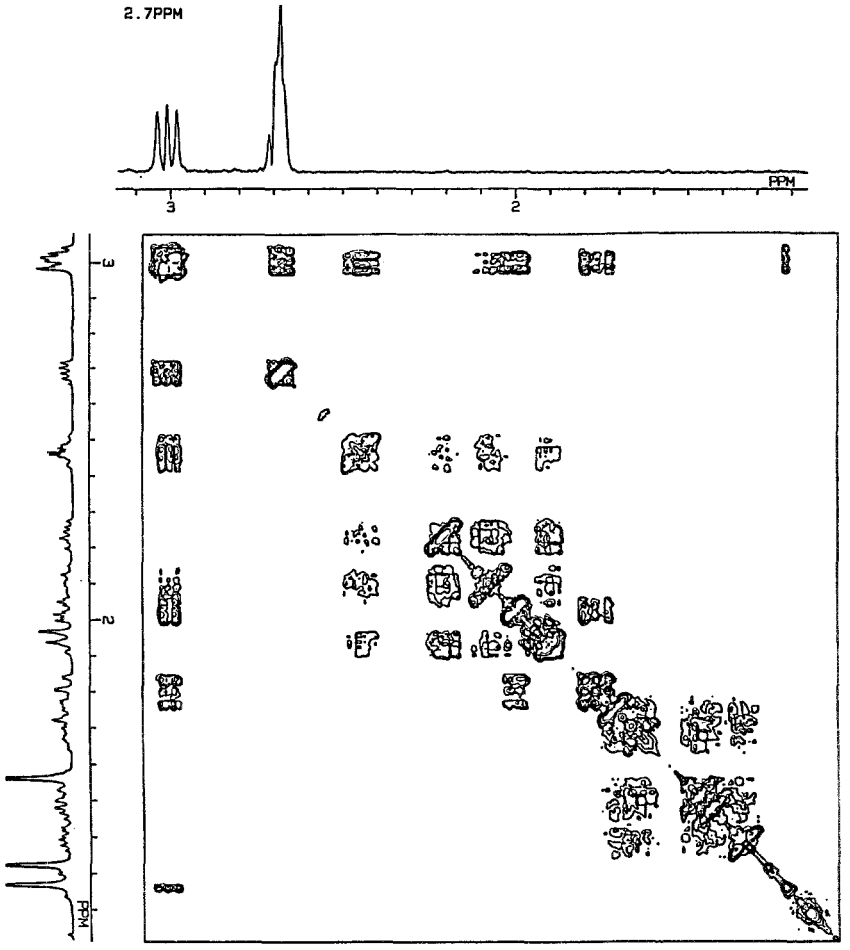


FIG. 3. [^{1}H]–[^{1}H] double quantum filter COSY and sliced spectrum at δ 2.70 ppm (H-12β) of ajugasterone C, **3**; 400 MHz, C_5D_5N. The δ 2.70 ppm signal would not be shown as the exact signal pattern.

several other insect pests. The details of the bioassay result will be reported elsewhere.

DISCUSSION

In general, it is still very difficult and time consuming to separate the side-chain isomers of any steroids. The R-HPLC technique was remarkably useful in their separation. The slice technique in the ^{1}H–^{1}H COSY spectrum was very

useful for observing individual protons overlapped with other protons and to determine the whole structure of the steroid derivatives. The ambiguity of the stereochemistry of ajugasterone C was resolved.

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FLIGHT AND LANDING BEHAVIOR OF *Trypodendron lineatum* (COLEOPTERA: SCOLYTIDAE) IN RESPONSE TO DIFFERENT SEMIOCHEMICALS

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Abstract—The responses of the striped ambrosia beetle, *Trypodendron lineatum* (Olivier) (Coleoptera: Scolytidae), to modified drainpipe traps baited with the host attractant ethanol, the aggregation pheromone lineatin, both alone and in combination, were studied in a wind tunnel. Exposure of males to ethanol increased their frequency of steady, upwind flight; however, only lineatin was effective in inducing them to land on and enter the traps. In contrast, exposure of females to either ethanol or lineatin alone resulted in an increased frequency of trap landing and entry. Both compounds released simultaneously did not significantly increase the frequency of trap landing and entry for either sex. These responses are consistent with the life history strategies used by both sexes to seek and colonize suitable host material.

Key Words—*Trypodendron lineatum*, ambrosia beetle, Coleoptera, Scolytidae, semiochemicals, flight behavior, wind tunnel, drainpipe trap.

INTRODUCTION

The effect of olfactory stimuli on forest Coleoptera is best understood for the bark and ambrosia beetles (Scolytidae), where orientation to host material is generally considered a set of sequential events (Borden et al., 1986; Schlyter et al., 1987). For the striped ambrosia beetle, *Trypodendron lineatum* (Olivier) (Coleoptera: Scolytidae), it is hypothesized that the pioneer females cue into the host attractants (primary attractant phase) and, upon successfully boring into

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a suitable log, emit a pheromone that results in the mass attack of the logs by males and females (secondary attraction phase) (Borden, 1985). The main host attractant for North American *T. lineatum* has been identified as ethanol (Moeck, 1970, 1971). The aggregation pheromone has been identified to be the tricyclic ketal, lineatin (3,3,7-trimethyl-2,9-dioxatricyclo[3.3.1.$0^{4,7}$]nonane) (MacConnell et al., 1977; Borden et al., 1979).

Over the past decade, development of a facile synthesis for the pheromone, along with the evolution of trap technology (Lindgren, 1983; Lindgren et al., 1983), has led to semiochemical-based pest management strategies around log processing areas (Borden, 1990). In an effort to increase efficiency of this program, investigators have looked at the response of *T. lineatum* to various combinations of host attractants and its pheromone (Borden et al., 1982; Salom and McLean, 1988). Interspecific response of *T. lineatum* and the closely related *Gnathotrichus spp.*, to their respective pheromones and host attractants, also has been investigated (Borden et al., 1981; Shore and McLean, 1983). Host attractants were concluded to be necessary components of the semiochemical bouquet needed for attracting North American *T. lineatum* by Shore and McLean (1983), whereas they were not in two other studies (Borden et al., 1982; Salom and McLean, 1988). This has resulted in some confusion as to the actual roles of these chemicals in North American *T. lineatum* host finding, orientation, and attack. Additionally, studies concerning *T. lineatum* in Europe have consistently shown that beetles respond to host attractants alone (Bauer and Vité, 1985) and respond synergistically to lineatin when host attractants are added to the bouquet (Borden et al., 1982; Paiva and Kiesel, 1985; Vité and Bakke, 1979), regardless of the types of traps employed for capturing beetles (Borden et al., 1982; McLean et al., 1987).

In this paper we investigate responses of *T. lineatum* from North America to traps baited with its pheromone, lineatin, and/or the host attractant, ethanol, in a wind tunnel. Our results are discussed with regard to the controversy over the essentiality of ethanol in the semiochemical bouquet of *T. lineatum*.

METHODS AND MATERIALS

Wind Tunnel. The wind tunnel used in this study (Angerilli and McLean, 1984), 3.6 m long and 1.2 m high and wide, has since been fitted with a dust and activated-charcoal filter. Cool white fluorescent lights (660 W), set 1.5 m above the wind tunnel, simulated diurnal conditions and facilitated direct observations of flying beetles. A sheet of cellulose acetate was placed over the top of the tunnel to diffuse the incoming light and reduce glare. Incident illumination readings from within the tunnel were measured with a Sekonic model L-398 exposure meter. Vertical light measured 0.8 m from the ceiling of the tunnel averaged 130 lx. Within the tunnel, temperature averaged 23 $\pm$ 2°C

(Rudinsky and Daterman, 1964), and wind speed was maintained at 15 cm/sec, for optimal beetle response within an airflow (Salom, 1989).

Beetles. Beetles were collected daily during May and June of 1987 and 1988, in lineatin-baited multiple funnel traps (Lindgren, 1983), near a large log boom storage area at the mouth of the north arm of the Fraser River, in Vancouver, British Columbia. Captured beetles were stored at 4°C under a 14 : 10 hr (light–dark) photoperiod, in 1-liter plastic jars containing slightly moistened absorbent cloth towels. The containers were checked twice weekly to monitor moisture levels. Beetles used in these experiments were not stored any longer than 50 days, although they were capable of surviving for more than 100 days under these conditions. Since the beetles were captured in pheromone traps, they were judged to have had sufficient flight exercise to ensure subsequent response to semiochemicals (Graham, 1959; Bennett and Borden, 1971).

Traps. Three Norwegian drainpipe traps (Borregaard Ind. Ltd., 1701 Sarpsborg, Norway) were equally spaced across the upwind sector of the wind tunnel, ca. 0.6 m from the screen. This trap design was chosen over other designs because it was considered to best simulate a log. External ridges, projecting 3 mm from the trap surface, circle the full length of the trap concentrically, allowing beetles to land and subsequently walk on the trap, as they would on a log. Numerous holes (3 mm diam.), punched between the ridges, simulate gallery holes in a log. They provide: (1) the openings needed for semiochemical emission and (2) entrances into the trap for responding beetles. As a result, beetles that land on these traps are required to go through a searching phase to find the semiochemical source, similar to a field situation where males search for mates and females search for suitable boring sites (McLean et al., 1987; Vité and Bakke, 1979).

Several modifications were made on these traps from those used in the field (Figure 1). Traps were painted a light green–gold color to allow for easier observation of beetle movement near and on the trap. Lindgren et al. (1983) found no differences in response by *T. lineatum* to funnel traps of five different colors, suggesting that painting our traps would not alter beetle response. The bottom section was fitted with a funnel that tightly connected the trap and the collecting jar. The semiochemical plume emitted from the trap was simulated using $TiCl_4$ smoke. Initial tests indicated that a funnel inserted into the collecting jar and the trap cover lifted 1 cm above the top of the trap achieved emission of the plume over the full length of the trap (Figure 1A). The semiochemical dispensers were placed inside the trap by a 1-cm-diam. wooden dowel suspended from the trap cover (Figure 1B). The length of each trap was 1.1 m, producing a composite plume from the three traps that covered most of the volume within the tunnel. Studies in this wind tunnel have indicated that *T. lineatum* respond at higher frequencies to tunnel-wide plumes of semiochemicals than to point source plumes of semiochemicals (Salom, 1989). This is con-

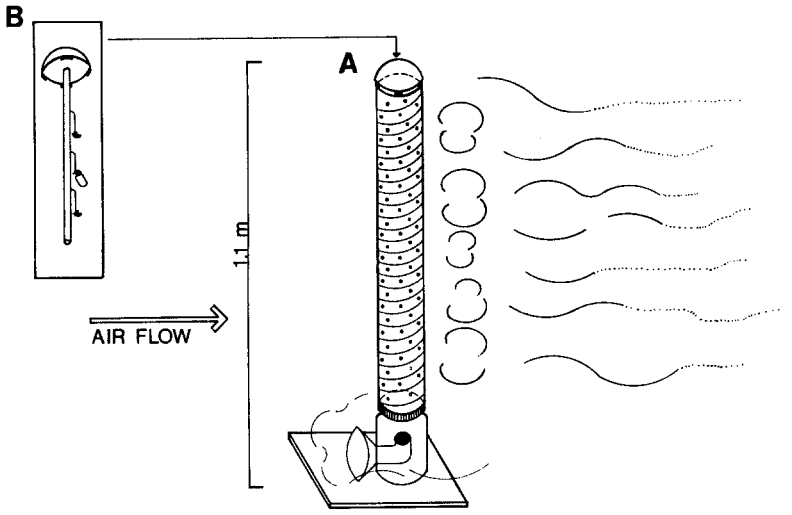


FIG. 1. (A) Modified Norwegian drainpipe traps used as a baiting substrate for *Trypodendron lineatum*, with a typical smoke plume pattern produced at 15 cm/sec. B) Close-up of trap cover and lure holder, showing an upper and lower lineatin bait and a medial ethanol bait.

sistent with sources of attraction used by the beetles in the field, where plume sizes may be as large as the volume of a group of dead trees or logs.

Treatments. Unbaited traps (control) and traps baited with ethanol and lineatin, alone and in combination served as the four treatments of study. In treatments containing alcohol, 95% ethanol was released from two 40-ml dispensers (total release rate = 150 mg/24 hr) (Phero Tech Inc., Vancouver, British Columbia), each placed in the center of the two outer traps. In treatments containing lineatin, the pheromone was dispensed from Hercon controlled release lures (Kydonieus and Beroza, 1981), where release rates were based on lure size (7 μg/24 hr/cm^2) (Phero Tech). Two 0.76-cm^2 lineatin lures were placed in the top and bottom of all three traps, resulting in a total release rate of 32 μg/24 hr (Salom and McLean, 1988). All lures were aged one week at ambient temperatures to allow release rates to stabilize. Ethanol could only be placed in two traps because of the high elution rates of the dispensers used. With the placement of three traps across the tunnel, sufficient turbulence occurred to produce reasonable mixing of the plume. No noticeable differences in beetle landing frequencies were observed between traps.

Traces of semiochemicals may have remained in the wind tunnel during the 1987 experiments; therefore, control treatments were tested again in 1988. Prior to the second run of control treatments, the wind tunnel was cleaned with

a 0.5% hypochlorite solution, the charcoal filter was reactivated, and the dust filter was replaced. New drainpipe traps were used also. All subsequent procedures described below were the same for both the first (all treatments) and second (controls only) experimental sets.

Test Procedure. A release platform consisting of a circular, flat glass plate (13 cm diam.), was suspended horizontally 35 cm above the tunnel floor and 1 m from the downwind screen. The plate was covered with a white industrial paper towel to enhance traction for the insects. A 16-cm^2 × 0.7-cm deep plastic weighing boat, covered by the same material, was taped on the upper side of the glass plate. This allowed the beetles two perching opportunities: the first around the edge of the weighing boat and, if they fell off that, the second around the edge of the glass plate.

Beetles were sexed and given a walking test in the morning prior to flight in the wind tunnel. The test required that the beetles walk normally, and that legs and antennal appendages be intact. They were allowed to acclimate to the warmer room temperatures (22–25°C) for 15 min before release, a period sufficient to ensure maximal response of the beetles to the traps (Salom, 1989).

Two beetles, with males and females tested separately, were released for each replication. All treatments were selected randomly within each replication. In 1987, one to four replications were conducted per day over 24 experimental days, whereas in 1988, 8–12 replications of the second set of unbaited treatments were conducted over six experimental days. In total, 124 beetles of each sex were tested in each treatment.

The detailed observations of beetle response to the different treatments included occurrence of flight, time to flight take-off, take-off direction, flight type, overall flight direction, lock-on behavior, landing on the traps, time to landing, and trap entry. Flight take-off direction was scored as upwind, vertical, or downwind. Type of flight was scored as steady—a settled, stable movement typical of flight in the field (personal observation), or erratic—quick, sharp changes in movement, often occurring when beetles bounced off the ceiling of the tunnel, probably in response to the overhead lights (Graham, 1959; Francia and Graham, 1967). Overall flight direction was scored as upwind, downwind, or nondirectional and was based on the predominant direction flown by the insect throughout the observation period. If predominantly up- or downwind flight was not exhibited by the beetles, flight was considered nondirectional. These categories were based on the amount of time a beetle spent in each section of the tunnel. Lock-on behavior can be described by steady flight in an upwind direction, with the insect orienting to the traps, either by casting up and down tunnel, hovering around the trap, or landing on it. For beetles landing on the traps, observations were made to see if they entered the traps. All observations were recorded on a cassette tape and later transcribed to data sheets.

Response behavior data were summarized as frequencies and analyzed sep-

arately for each behavioral category, as multidimensional contingency tables using the CATMOD procedure in SAS (1985). This procedure uses a linear models approach to the analysis of categorical data, applying general weighted least-squares regression techniques to estimate cell proportions in complex categorical data layouts (Kleinbaum and Kupper, 1978). This approach allowed us to test for significant interactions and main effects and to contrast between levels of main effects for a specific response, using the chi-square (χ^2) statistic. Response time data were analyzed using General Linear Model (GLM) (SAS, 1985), with subsequent mean separation between treatments carried out with the Student-Newman-Keul's test ($P \leq 0.05$).

RESULTS

No significant differences were found between the first- and second-year unbaited control treatments for all behaviors and time responses observed, except for flight takeoff direction (Table 1). The different results in flight takeoff direction did not apparently affect any other set of results. Therefore, the unbaited treatment results from year 1 (1987) are considered a valid control for all behavior categories studied except flight take-off direction. In the latter case, both year 1 and year 2 (1988) unbaited treatment results are presented.

Occurrence of flight differed significantly between males and females (χ^2_1

TABLE 1. STATISTICAL COMPARISON OF *Trypodendron lineatum* RESPONSES TO FIRST- AND SECOND-YEAR CONTROL TREATMENTS IN WIND TUNNEL[a]

Behavior observed	Test statistic (degrees of freedom)[b]	Probability of significance level
Occurrence of flight	$\chi^2_1 = 0.69$	0.4054
Time of flight takeoff	$F_{1,283} = 0.01$	0.9094
Flight takeoff direction	$\chi^2_2 = 8.33$	0.0155
Flight type	$\chi^2_1 = 0.00$	0.9451
Overall flight direction	$\chi^2_2 = 2.10$	0.3495
Lock-on response	$\chi^2_2 = 0.98$	0.6111
Time between takeoff and landing	[c]	[c]
Captured in traps as % of beetles locking-on	$\chi^2_1 = 0.00$	0.9491

[a] The numbers of beetles released for each treatment = 248.
[b] Degrees of freedom for the χ^2 statistic are a product of treatment ($t - 1$) by response levels ($r - 1$).
[c] Not enough responders to make comparisons.

= 5.7; $P \leq 0.05$), averaging 62% and 69%, respectively. However, no significant differences were observed among semiochemical treatments ($\chi^2_3 = 6.7$; $P > 0.05$). Response time between beetle release and initial flight was found to be independent of treatment and sex ($F_{9,764} = 0.8$; $P > 0.05$). Mean takeoff time for all treatments averaged 80 sec.

Differences in upwind and vertical takeoff directions were observed between the first- and second-year unbaited controls (Figure 2). Upwind takeoff was higher in year 1 and vertical takeoff was higher in year 2 treatments. In year 1, no differences in upwind takeoff direction were observed for any treatments. An increase in downwind takeoff was observed in the presence of lineatin and ethanol together.

Type of flight was influenced by the presence of semiochemicals, yet no differences were observed between males and females ($\chi^2_1 = 0.2$; $P > 0.05$), therefore the data were pooled (Figure 3). The presence of ethanol resulted in a significant increase in steady flight from 27% to 37% when compared to the unbaited treatment. With lineatin present, the frequency of steady flight

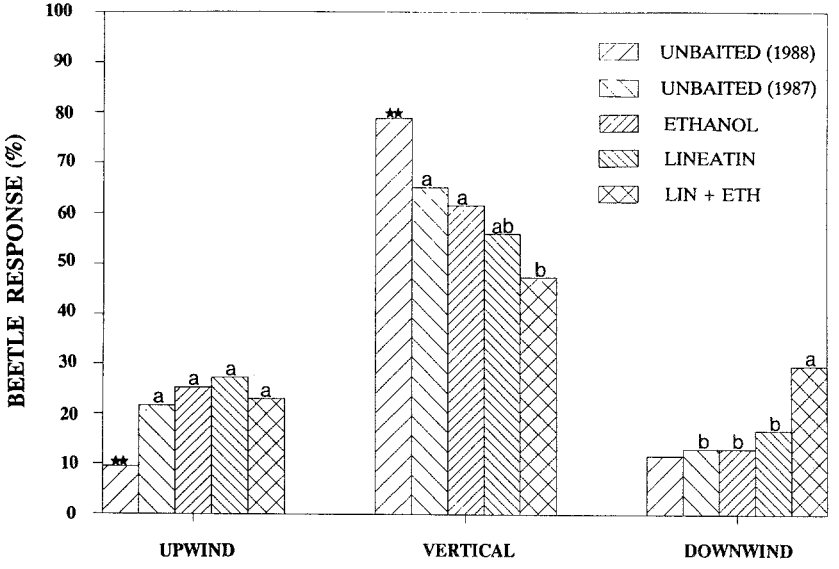


FIG. 2. Flight takeoff direction of *Trypodendron lineatum*, in the presence of semiochemical baits in drainpipe traps, within a wind tunnel. Male and female data were pooled. Stars on top of unbaited (1988) bars denotes a significant difference (χ^2; $P \leq 0.05$) with the unbaited (1987) treatment. Bars for each direction with different letters indicate significant differences in response (χ^2; $P \leq 0.05$) for individual comparisons. The numbers of observations for each treatment were: unbaited (1988), 137; unbaited (1987), 138; ethanol, 146; lineatin, 150; and lineatin and ethanol, 152.

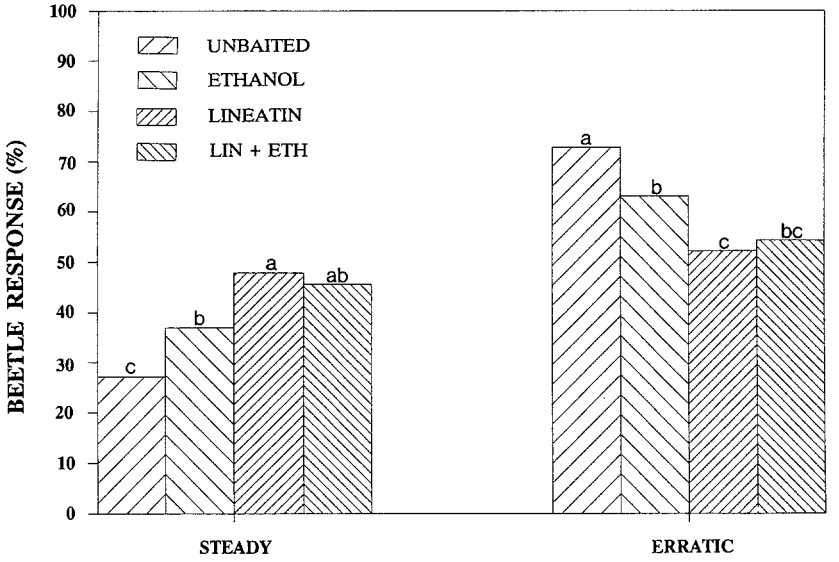


FIG. 3. Observations of flight type exhibited by *Trypodendron lineatum*, in the presence of semiochemicals in drainpipe traps, within a wind tunnel. Male and female data were pooled. Proportions are based on the total number of observations, where each individual could be scored for more than one type of flight. Bars for each type of flight with different letters indicate significant differences in response (χ^2; $P \leq 0.05$) for indvidual comparisons. The numbers of observations for each treatment were: unbaited, 140; ethanol, 152; lineatin, 158; and lineatin and ethanol, 156.

increased significantly to 48%. Ethanol combined with lineatin did not further increase the frequency of steady flight.

Overall flight direction was influenced by the presence of semiochemicals, yet no differences were observed between males and females ($\chi^2_2 = 0.9$; $P > 0.05$), therefore the data were pooled (Figure 4). Upwind flight direction increased significantly from 15% to 31% in the presence of ethanol alone when compared to the unbaited traps and was offset by a decrease in downwind flight from 67% to 50%. Further increases in upwind flight, from 31% to 45%, occurred in the presence of lineatin alone. While this difference was not significant, the resulting decrease in nondirectional flight was. Responses to ethanol and lineatin combined did not differ from responses to lineatin alone.

Lock-on response patterns between sexes differed upon exposure to the different semiochemical treatments (Figure 5). The landing of males on drainpipe traps, following lock-on response, was not influenced significantly by the

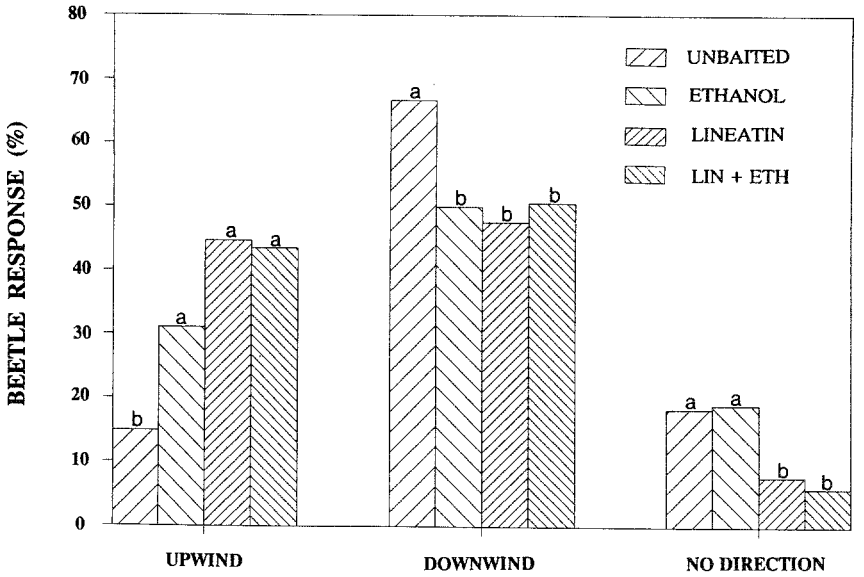


FIG. 4. Overall flight direction exhibited by *Trypodendron lineatum*, in the presence of semiochemicals in drainpipe traps, within a wind tunnel. Male and female data were pooled. Bars for each direction with different letters indicate significant differences in response (χ^2; $P < 0.05$) for individual comparisons. The numbers of observations for each treatment were: unbaited, 141; ethanol, 158; lineatin, 166; and lineatin and ethanol, 166.

presence of ethanol; however, with lineatin present, landing frequency increased from 2% to 27%. The number of beetles landing did not increase significantly when lineatin and ethanol were combined. The frequency of males exhibiting lock-on behavior without landing did not change for any baited treatment.

The frequency of females exhibiting lock-on response, followed by trap landing, was significantly greater in the presence of ethanol when compared to the unbaited treatment, with an increase in response from 1% to 12% (Figure 5). The presence of lineatin also significantly enhanced landing, with 16% of the flown females responding. The combination of ethanol with lineatin did not result in an increased response over lineatin or ethanol alone. Lock-on response without landing increased slightly from 18% to 24% with ethanol present and significantly increased over the unbaited control to 32% when only lineatin was present. The combination of ethanol and lineatin did not improve this response.

The time interval between beetle flight takeoff and landing on the trap also differed with semiochemical treatments, yet was independent of sex (Figure 6).

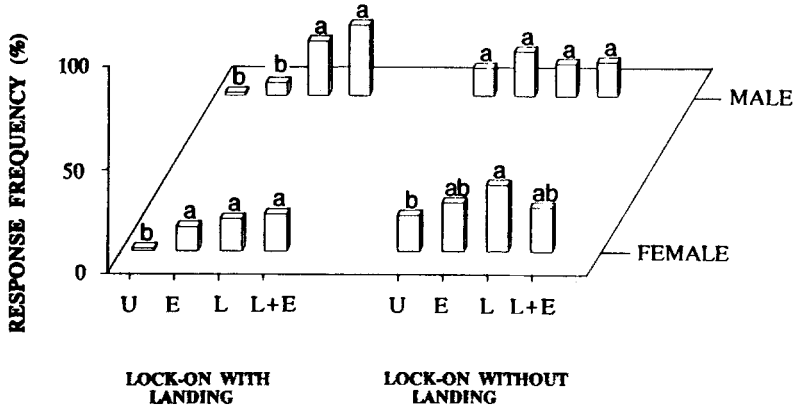


FIG. 5. Lock-on response, exhibited as landing or nonlanding behavior, by *Trypodendron lineatum* in the presence of semiochemical baits in drainpipe traps, within a wind tunnel. Bars for each behavior and sex with different letters indicate significant differences in response (χ^2; $P \leq 0.05$) for indvidual comparisons. The numbers of males observed for each treatment were: unbaited (U), 67; ethanol (E), 79; lineatin (L), 83; and lineatin and ethanol (L + E), 79. The numbers of females observed were: U, 80; E, 85; L, 90; and L + E, 89.

The small number of ethanol-only responders would make it difficult to detect statistical differences between sexes; therefore male and female response times are presented as separate bars. Time response to ethanol-baited traps was significantly shorter than to lineatin-baited traps, averaging 81 and 191 sec, respectively. Intermediate responses were observed when lineatin and ethanol were combined, with an average response time of 128 sec.

The presence of lineatin was critical in inducing males to enter the traps. With respect to the total number of beetles released, 12% were captured; of the beetles that flew, 18% were captured; and of the beetles that exhibited lock-on behavior, 43% were captured (Table 2). Responses to ethanol were not significantly higher than responses to unbaited traps, and responses to lineatin and ethanol together were not significantly higher than lineatin alone.

In contast, females were stimulated to enter the traps by the presence of ethanol and lineatin (Table 2). For traps baited with ethanol only, 7% of the total number of beetles released were captured, 9% of the number that flew were captured, and 28% of the number that exhibited lock-on behavior were captured. Responses to traps baited solely with lineatin were very similar to those baited with ethanol alone. The proportion of beetles entering the traps, with respect to all categories in Table 2, was higher when ethanol and lineatin

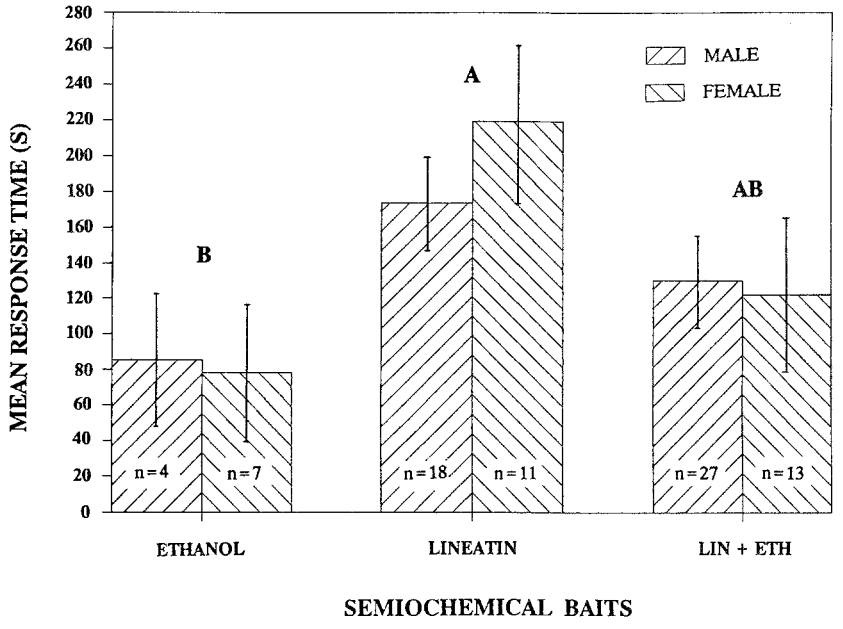


FIG. 6. Mean time interval ± SE (indicated by vertical lines running through the top of each bar) between flight takeoff and trap landing of *Trypodendron lineatum*, in the presence of semiochemicals in drainpipe traps, within a wind tunnel. Different letters on the top of the bars indicate significant differences between semiochemical baits (SNK; $P \leq 0.05$).

were combined; compared to ethanol or lineatin alone, however, this increase was only significant for beetles exhibiting lock-on behavior.

DISCUSSION

Flight initiation patterns, with respect to flight frequency and time of flight, were not affected by the presence of semiochemicals. Take-off direction could not be adequately described, because of the varying results obtained between the unbaited controls in years 1 and 2. However, no differences were found between control treatment responses for any other observed behavior (Table 1).

Steady flight increased and erratic flight decreased when beetles were exposed to semiochemicals (Figure 3), suggesting that flight type may have potential in describing *T. lineatum* response in wind tunnel studies. Steady flight may indicate that beetles are in a receptive mode to respond to attractants. Although some beetles responded positively following a period of erratic flight, most positive responses came from beetles that initially flew in a steady manner.

TABLE 2. CAPTURE OF *Trypodendron lineatum* IN SEMIOCHEMICAL-BAITED DRAINPIPE TRAPS IN WIND TUNNEL AS PERCENTAGES OF OBSERVATIONS FOR DIFFERENT BEHAVIORAL CATEGORIES RECORDED[a]

Attractant	No. beetles entering trap	Beetles released (%)	Beetles that flew (%)	Beetles locking on (%)	% of Beetles landing on a trap (%)[b]
Unbaited	1	0.8 b[c]	1.5 b	9.1 b	100.0
Ethanol	3	2.4 b	3.8 b	13.0 b	60.0
Lineatin	15	12.1 a	18.1 a	42.9 a	71.4
Lin + Eth	19	15.3 a	24.1 a	47.5 a	70.4
Females					
Unbaited	0	0.0 b	0.0 b	0.0 c	0.0
Ethanol	8	6.5 a	9.4 a	27.6 ab	80.0
Lineatin	7	5.6 a	7.8 a	16.3 b	50.0
Lin + Eth	13	10.5 a	14.6 a	37.1 a	81.3

[a] $N = 124$ released/treatment/sex.
[b] Individual comparisons between treatments could not be carried out because of low numbers of insects.
[c] For each column, within each sex, percentages followed by different letters are significantly different (χ^2; $P \leq 0.05$), for individual comparisons.

From field observations, we suspect that beetles exhibiting steady flight were unaffected by the wind tunnel, and those that exhibited erratic flight may have been responding to the unnatural conditions imposed upon the insects by a constrained environment.

Males and females exhibited similar overall flight direction patterns in the presence of semiochemicals (Figure 4). Both ethanol and lineatin were important in influencing upwind flight of the beetles, with only lineatin causing a decrease in nondirectional flight. While upwind flight response to ethanol was expected for females, as they are the pioneering sex for this species, it was unexpectedly important for males.

Male response to ethanol did not carry over to lock-on behavior, nor did it play a role in inducing them to land on the traps. In contrast, females required ethanol almost as much as lineatin to induce lock-on flight and trap landing behavior.

Interestingly, responsive beetles of both sexes landed on the traps in decreasing time in the presence of lineatin alone, ethanol and lineatin together, and ethanol alone (Figure 6). This provides additional evidence that ethanol plays a role not only in attracting females, but to a lesser extent, in attracting males to host material. For males, only lineatin induced trap entry, whereas for females, the presence of both ethanol and lineatin induced trap entry (Table 2).

Wind tunnel experiments have been conducted to determine the pretest physiological requirements of the beetles, wind speed (Salom, 1989), and semiochemical release rates (Salom and McLean, 1988) that elicit optimal responses of *T. lineatum* to olfactory stimuli. Additional studies that can further enhance this bioassay include testing *T. lineatum* flight response with a rolling floor in the tunnel to satisfy potential optomotor requirements (David, 1986) that may not have been met by the nonmovable striped floor in our study, the effect of varying lighting regimes illuminating the tunnel, and the addition of another possible host kairomone, α-pinene (Nijholt and Schonherr, 1976), to the semiochemical bouquet. Despite the need for further work in this area, the study reported in this paper was discriminating enough to demonstrate the relative importance of ethanol and lineatin in eliciting attraction to and arrestment of *T. lineatum* on a host substrate.

Overall, lineatin was more important than ethanol in influencing males to fly upwind to, land on, and enter the traps. For females, lineatin was more important in stimulating steady, upwind flight than was ethanol. However, both compounds were equally important in inducing trap landing. As a percentage of females exhibiting lock-on behavior, significantly more beetles were captured when both ethanol and lineatin were used together compared to lineatin alone. These results suggest that the host stimuli may serve as a close-range attractant and the pheromone as a long-range attractant. A similar hypothesis has been put forward for the host attractant and pheromone of the spruce bark beetle, *Ips typographus* L., based on electrophysiological studies (Dickens, 1981). It was found that *I. typographus* responds to its pheromone *cis*-verbenol at a lower as well as a wider range of concentrations than to concentrations of its host attractant α-pinene, indicating the insects' ability to perceive the pheromone at greater distances than the host volatiles.

The results of male and female *T. lineatum* responses to semiochemicals in the wind tunnel differ from field studies performed by Borden et al. (1982). These authors found that the capture of both males and females in drainpipe traps was inhibited by the presence of ethanol. However, the release rates of ethanol in the experiments were almost seven times higher than those reported here, not including additional ethanol volatiles emitted from nearby logging slash. In fact, they reported that high release rates of ethanol, at 1 g/24 hr, inhibited male response when compared to lower release rates, at 120 mg/24 hr. In contrast, Shore and McLean (1983) reported increases in male and female catches to drainpipe traps baited with ethanol, α-pinene, and lineatin, when compared to traps baited solely with lineatin. However, Shore and McLean (1983) did not test ethanol and α-pinene separately. Salom and McLean (1988) determined in both field and laboratory experiments that neither ethanol nor α-pinene were important in capturing *T. lineatum* in multiple funnel traps. Yet beetles must enter holes for capture in drainpipe traps, and lack of this behavior

requirement in funnel traps may account for the difference in the results between the two studies.

This study reports the first detailed observations describing the flight behavior of *T. lineatum* in the presence of semiochemicals. Using optimal release rates of ethanol and lineatin (Salom and McLean, 1988), the results support the hypothesis that pioneering females rely partially on the host attractant ethanol, while males, although not inhibited by ethanol, appear to rely mostly on lineatin in searching for female mates. Ethanol appears to reduce the time of response for both males and females (Figure 6), resulting in less exposure of these beetles to predators and other mortality agents during their spring dispersal flight. If males were as attracted to ethanol as females are, they would possibly find alternative host material instead of homing in on tunneling females who are ready to mate and produce brood needed to perpetuate the population. While males cannot afford to search for potential suitable hosts, and take the chance of not finding a mate, females can afford to respond to alternative but equally suitable host material, since they produce the aggregation pheromone.

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BEHAVIORAL RESPONSES OF MALE *Periplaneta americana* L. TO FEMALE SEX PHEROMONE COMPONENTS, PERIPLANONE-A AND PERIPLANONE-B

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Abstract—Bioassays were performed on male *Periplaneta americana* L. using synthetic (−)-periplanone-B (P-B) and Hauptmann's (−)-periplanone-A (P-A), and their mixtures at various ratios to estimate the roles of both periplanones for the sexual communication of the species. Both P-A and P-B elicited qualitatively the same responses, such as antennal movement, rapid locomotion, wing raising, and homosexual behavior of male cockroaches, but the threshold of the pheromone activities for P-B was two orders of magnitude lower than that of P-A. Neither synergistic nor inhibitory but only a simple integrated effect on the responses was observed when mixtures of P-A and P-B were applied.

Key Words—*Periplaneta americana*, sex pheromone, Orthoptera, Blattidae, periplanone-A, periplanone-B, American cockroach.

INTRODUCTION

The major sex pheromones so far isolated from the female American cockroach, *Periplaneta americana* L. are periplanone-A (P-A) and periplanone-B (P-B). Quite recently, isolation and identification of periplanone-D_1 and periplanone-D_2 were reported as new sex pheromone components (Biendl et al., 1989). The

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structures of P-A and P-B were proposed on the basis of spectroscopic data (Fig. 1) (Persoons et al., 1976, 1979, 1982; Talman et al., 1978). In the case of P-B, the proposed structure was confirmed by synthesis (Still, 1979), and the complete structure, including the absolute stereochemistry for P-B, was established by Adams et al. (1979) and by Kitahara et al. (1987). The proposed structure of P-A has not been settled to date. One of the two possible diastereomers of Persoons' P-A was synthesized and found to be different in its spectral data from natural P-A and had no biological activity. The other diastereomer also differed from the reported spectral data (Shizuri et al., 1987). Recently, Hauptmann et al. (1986) reported the isolation and structural elucidation of a new sex pheromone component and the structure was confirmed by synthesis. This compound may be one of the major sex pheromones of the American cockroach and is called Hauptmann's P-A (Figure 1).

The origin and the roles of P-A and P-B in the sexual communication of this species have been investigated, but the results obtained have been ambiguous because enantiomeric mixtures of synthesized samples (Tobin et al., 1981) or active fractions (Seelinger, 1985; Seelinger and Gagel, 1985) have been used. Talman et al. (1978) suggested P-A to be one of the breakdown products of P-B developing in the feces. To the contrary, Sass et al. (1983) suggested that the P-A was not the degradation product of P-B but a natural pheromone with biological significance, based on electrophysiological evidence using purified extracts. As to the roles of P-A and P-B, it is suggested that P-B is responsible for long-distance attraction, while P-A influences on male behavior near the female (Seelinger, 1985).

Synthetic Hauptmann's (−)-P-A and (−)-P-B, which are identical to the natural components, are now available; this enables us to evaluate the roles of (−)-P-A and (−)-P-B in sexual communication of *P. americana* without the complications of enantiomeric mixtures (Mori et al., 1986; Kodama et al., 1987; Yamada et al., 1976).

The purpose of this paper is to investigate the pheromonal responses of *P. americana* to Hauptmann's P-A and P-B using synthetic optical active compounds.

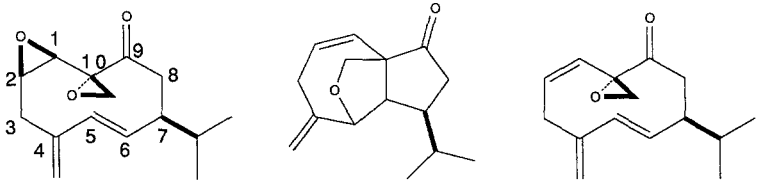


FIG. 1. Structures of sex pheromone components produced by female *P. americana*.

METHODS AND MATERIALS

Insects. Male cockroaches from the institute's colonies were selected before imaginal ecdysis and kept in a room that was isolated from females and female odor. Water and commercial dry mouse food (Charles River Japan Company) were provided ad libitum. Room temperature was 25 ± 1°C and relative humidity was 60 ± 10%. Light regime was a 12 : 12 light–dark photocycle, dark phase starting at a 1200 hr. All experiments were performed during the first 5 hr of the dark phase, which was reported to be the active period of the male *P. americana* (Hawkins and Rust, 1977). Males used for this experiment were normal, with intact antennae and legs, in weeks 4–12 of their adult life. A red photographic light was used for illumination at intensities lower than 5 lux.

Pheromone Sources. P-B used in this experiment is optically active ($[\alpha]_D^{25}$ − 557°, c = 0.113, n-hexane) and has 1*R*, 2*R*, 5*E*, 7*S*, 10*S* absolute configuration (Kitahara et al., 1987). Hauptmann's (−)-P-A is also optically active ($[\alpha]_D^{25}$ = −968°, c = 0.025, n-hexane) (Mori et al., 1988). Spectroscopic properties of these two synthetic compounds were identical to those of naturally occurring P-A (Hauptmann et al., 1986) and P-B (Persoons et al., 1976). Although the chirality at C-10 in P-A is unknown yet, the synthetic P-A is considered to be identical to the natural compound because of complete coincidence of high-field [^{1}H]NMR spectral data with that of natural P-A reported by Hauptmann et al. (1986), and the *S*-configuration at C-7, which was derived from a known optically active starting material. These two pheromones are crystalline compounds (P-A: mp 43.5–45°C, P-B: mp 57–57.5°C), and chemical purities of these synthetic pheromones were confirmed to be over 99.9% by high-performance liquid chromatography.

Behavioral Assays. All behavioral assays were conducted in a clean glass assay box that could be taken apart to clean (Figure 2). The volume of the assay

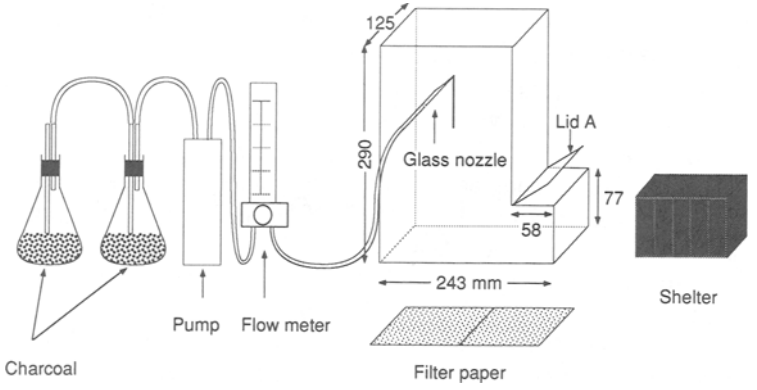


FIG. 2. Diagram of the behavioral bioassay system.

box is approximately 7.4 liters. Mineral oil (heavy white oil, Sigma) was applied to the walls to keep the tested cockroaches on the floor. The odorants were injected via elbow-shaped glass nozzles made of a Pasteur pipet (Clay Adams No. 4647), the inner surface of which had been wetted with 1 μl pheromone solution diluted with *n*-hexane. The solvent was allowed to evaporate before the nozzle was used. One of the sidewalls of the assay box had a hole (6 mm diameter) through which the nozzle was fitted. The tip was set 18 cm above the bottom and oriented to the center area of the bottom. The odor substances were blown vertically down into the assay box with air twice purified through a charcoal filter (flow rate ca. 200 ml/min). Although the air in the assay box could be purged from a small hole (8 mm diameter) in the upper side of the assay box, the whole volume of introduced pheromones vapor (200 ml/min $\times$ 5 min = 1000 ml) was considered to remain in the assay box (volume 7400 ml) in the 5-min period of the experiment since the total volume of the assay box was big enough to contain the total volume of the airstream containing the pheromone vapor. To avoid contamination of the sample, glass nozzles were used only once, and all assay boxes used in the assay were dismantled for washing soon after the bioassay and before for the next use.

A tiny wood shelter box was previously put in a rearing cage to keep the cockroaches at rest. Before a behavioral bioassay, the shelter box with the five resting cockroaches was carefully taken from the rearing cage and introduced into the bioassay cage (lid A, Figure 2). This procedure avoided disturbing the resting cockroaches in the shelter box before the behavioral bioassay started. After finishing the test, the shelter box with the tested cockroaches was immediately returned to the rearing cage so as not to expose them further to the pheromone in the assay box and to avoid heavy habituation of males to high concentrations of pheromones. As the shelter boxes were scarcely polluted by the pheromones, they were not renewed thus offering accustomed circumstances to the insects used for the bioassay.

ACPM values for P-A, P-B, and Mixture of P-A and P-B. Sex pheromone activity was estimated by the total number of times the test male cockroaches crossed a line drawn across the center of the bottom of the assay box per minute, defined as ACPM (total activity counts per minute for the five insects) (Tobin et al., 1981). Measurement of ACPM was performed by 20 replications of the bioassay using five male cockroaches at each pheromone dosage. Dilution series of P-A at the dosage of 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2} μg (per 1 μl of distilled hexane) and of P-B at 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4} μg (per 1 μl of distilled hexane) were used for the behavioral bioassay. One microliter of distilled hexane was used as the control.

The bioassays of the mixtures of P-A and P-B were conducted by simultaneous introduction of the two components to the glass nozzle using two microsyringes. Pheromone responses to 12 combinations made from P-A at dosages

of 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4} μg and P-B at dosages of 10^{-7}, 10^{-6}, and 10^{-5} μg were measured to estimate whether a synergistic, inhibitory, or simple integrated effect occurred when mixtures of P-A and P-B were applied.

These diluted solutions of the synthetic pheromones were kept in a freezer until 20 min before the bioassay started. To avoid errors in counting, the responses of the insects were recorded by video tape recorder throughout the assay. Males were not tested more than twice a week because habituation reduced responsiveness (Seelinger, 1985).

RESULTS

Both (−)-P-A and (−)-P-B elicit typical sex pheromonal responses to male American cockroaches. The temporal sequence of responses such as antennal movement, rapid locomotion around the nozzle introducing the pheromones, wing raising, and homosexual behaviors could be elicited by both P-A and P-B. The threshold levels of the locomotory activity for P-B are considered to be 10^{-8} μg in the bioassay system (Figure 3). In this system, mean ACPM $\pm$ SD of the control is 1.5 $\pm$ 2.7. No significant ACPM of male *P. americana* for P-B at the dose of 10^{-9} μg was obtained, but antennal movement was observed at this dose.

The ACPM values for P-B increased in the range from a dosage of 10^{-8} to 10^{-4} μg except the ACPM value for 10^{-4} μg at the first minute. The ACPM values for P-B at 10^{-4} μg at the first minute indicated that saturation to the pheromone stimulus occurred. The maximal ACPM value for P-B was 102.8 at a dosage of 10^{-5} μg at the first minute in the bioassay period.

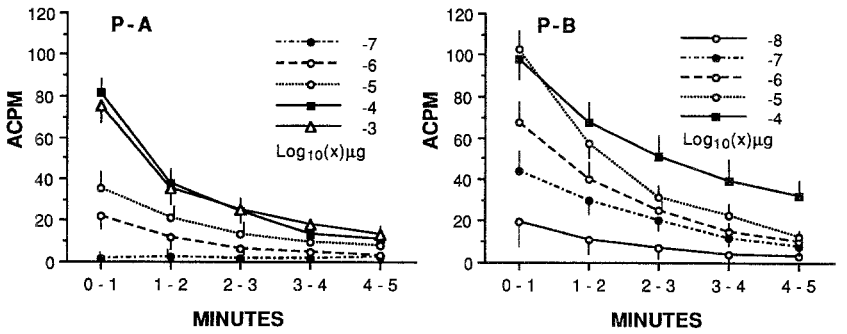


FIG. 3. Response of male *P. americana* to (−)-P-A and (−)-P-B. Responses are expressed as ACPM, the activity counts per minute for five males. Each point represents the mean of 20 replications and vertical bars across the data points indicate standard error.

The threshold of the locomotory activity for P-A is considered to be 10^{-6} μg. An ACPM value for P-A at 10^{-8} μg and a significant ACPM value at 10^{-7} μg were not observed, although antennal waving of the male for P-A was observed. ACPM values for P-A increased in the range from the dosage of 10^{-6} μg to 10^{-4} μg. Saturation for P-A was observed at the dosage of 10^{-3} μg. The maximal ACPM value for P-A was 81.7 at 10^{-4} μg for the first minute.

Thus, the threshold level of P-B for a significant increase of the locomotary activity is two orders of magnitude lower than for P-A. The maximal ACPM value of P-B is significantly greater than that of P-A (significant level 5% Student's t test). The range of P-B, from 10^{-8} to 10^{-4} μg, for significant increase of the response is much wider than that of P-A, from 10^{-6} to 10^{-4} μg.

The ACPM values for each dosage of P-A and P-B at the first minute in the 5-min assay can be related to the log of the pheromone amount to give two regression equations: $Y_{pa} = 25.5 \log_{10} (X_{pa}) + 175.5$ for P-A ($r^2 = 0.933$ interval 10^{-7} μg to 10^{-4} μg) and $Y_{pb} = 27.3 \log_{10} (X_{pb}) + 235.8$ for P-B ($r^2 = 0.989$ interval 10^{-8} μg to 10^{-5} μg) (Figure 4).

To estimate the effects of addition of P-A to P-B, a behavioral bioassay was conducted using samples with 12 mixture combinations. The observed ACPMs for the mixed samples of P-A and P-B were compared with the expected ACPMs, which were calculated by the above two regression equations for P-A and P-B, on the assumption that both P-A and P-B act as sex pheromones independently and elicit only the integrated effect, without any synergistic or inhibitory effects when mixtures of P-A and P-B are applied (Figure 3).

The expected ACPM values for the mixture of X_a μg of P-A and X_b μg of P-B can be obtained from the regression equation of P-B:

$$Y_{pb} = 27.3 \log_{10} (X_{pb}) + 235.8 \quad (1)$$

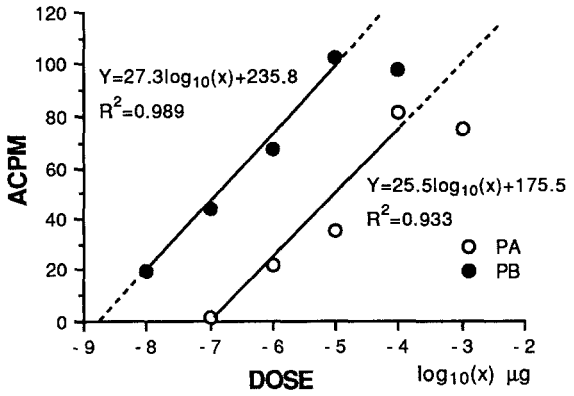


FIG. 4. Regression lines of dose–response for P-A and P-B. Data used in the calculation are the ACPMs of each dose at the first minute.

by the substitution of $X_{a'} + X_b$ for X_{pb}, where $X_{a'}$ is the equivalent amount of P-B to the X_a μg of P-A. $X_{a'}$ is obtained by the substitution of Y_{X_a} (which is an expected ACPM value of X_a μg P-A) for Y_{pb} into equation 1. Y_{X_a} is calculated by the substitution of X_a for X_{pa} into the regression equation of P-A: $Y_{pa} = 25.5 \log_{10}(X_{pa}) + 175.5$. There is little difference among the expected values of the P-A addition series to P-B 10^{-5} μg, which are obtained by the above procedure because they are dominated by the high dosage of P-B (Figure 5). Statistical differences between these expected and observed ACPM values were analyzed by Student's t test (significant level 5%) to estimate the effects of the addition of P-A to P-B (Figure 5). This comparison study indicated that no significant difference exists between the observed and the expected ACPM of the mixture.

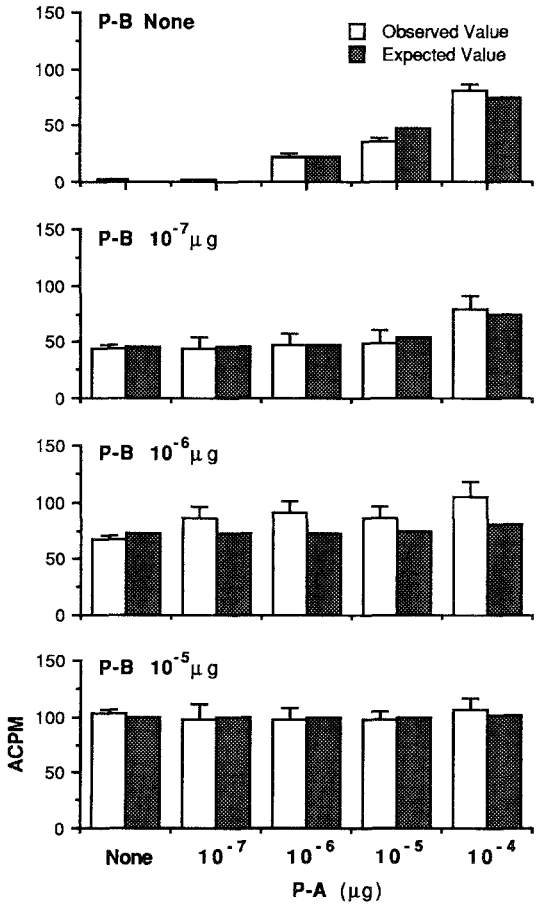


FIG. 5. Response of male *P. americana* to the mixture of P-A and P-B. The vertical bar indicates the standard error of the mean.

Consequently, no special ratio of the mixture of P-A and P-B that suppressed or reinforced the activity of P-B could be found, but the addition of P-A to P-B elicited only an integrated pheromone response.

DISCUSSION

It is most important to develop a reliable assay system for accurate evaluation of biological activities of possible pheromone compounds. We tried two systems in order to evaluate pheromone activities of compounds to the American cockroach. Preliminary results showed that, when filter paper impregnated with the test compounds was suspended from the ceiling of the assay box where American cockroaches had been released or when put on the bottom of the assay box, irregular time lags were observed before the cockroaches reacted to the pheromones and that the responses were weak for the first 1–2 min before maximal ACPM values were observed. Because of the slow release rate of the pheromones from the filter paper, it seems to take a few minutes for the concentration of the pheromones to elicit a maximal behavioral response. Our method, in which assay samples were introduced into the assay box with fresh air, is similar to a flowing air-delivery system described by Seelinger (1985). This enabled us to observe reliable and steep rises of responses to each concentration of pheromones. Installation of the shelter in the assay box kept cockroaches calm and reduced escape behavior locomotion. These improvements allowed us to get reliable ACPM values at various doses, especially at the threshold dose, where it is difficult to distinguish locomotion attributed to the pheromone from that of escape behaviour.

Electrophysiological studies suggested that the "active fraction called P-A" and P-B are perceived in different receptor cells in the male antenna and that there are specific interneurons for P-B and "active fraction called P-A" in the deutocerebrum (Burrows et al., 1982). This suggests a specific function for the "active fraction called P-A." P-A releases the same behavior in males as does P-B in a number of different behavioral assays (Seelinger 1985). In our experiments, using synthetic pure components, no difference was observed in the behavior elicited by P-A and P-B in terms of the series of courtship behavior, antennal waving, erect body posture, running, wing-raising, wing-fluttering, and courtship displays towards other males. It is reported that cockroaches habituated faster to P-B than P-A in the activity assay and that analysis of the orientation responses by individuals to a pheromone source show differences in the orientation behavior to P-A and P-B (Seelinger, 1985; Seelinger and Gagel, 1985). The difference in the habituation and the orientation behavior was not clear in our assay, but the decline of ACPM, which may represent habituation, for P-A seems to be faster than that for P-B during a 5-min bioassay.

The threshold values of P-A and P-B were defined to be the doses giving a significant increase of ACPM in the first 1-min interval, compared to that of the control experiments (Student's *t* test, significant level 5%). The locomotory threshold levels for P-A and P-B were 10^{-6} μg and 10^{-8} μg, respectively, but the insects apparently detected below ACPM threshold doses as they moved their antennae more frequently than usual at doses one-tenth lower for both pheromones.

The actual airborne ratio of P-A and P-B released from the female is unknown, although the ratio of P-A and P-B in the feces and dirtied filter paper was found to be 8:5 (Nishino et al., 1988). Because both P-A and P-B have similar chemical and physical properties, they may evaporate and diffuse into the air in the same way. P-B, whose locomotory threshold is two orders of magnitude lower than P-A, might play an initial role in guiding the male cockroach in the search for females from a distant place. Whether P-A has a role of changing the biological activity of P-B on its mixing ratios was investigated, although neither inhibitory nor synergistic effects were observed. Therefore, the ratio is not critical to elicit behavioral response in this bioassay.

The major component of the "active fraction called P-A" used for the previous behavioral assays (Seelinger et al., 1985) and electrophysiological experiments (Burrows et al., 1982; Sass, 1983) has not yet been proved to be Hauptmann's P-A. Results of our experiments are in good agreement with those obtained by Seelinger using the fraction, so the major component may be Hauptmann's P-A, but further studies on the electrophysiology of the olfactory cells and the neurons in the deutocerebrum should be required for confirmation of Hauptmann's P-A being natural P-A.

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IDENTIFICATION OF SEX PHEROMONE OF BRISTLY CUTWORM, *Lacinipolia renigera* (STEPHENS)

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Abstract—The sex pheromone of the bristly cutworm moth, *Lacinipolia renigera* was identified as a blend of (*Z*)-9-tetradecenyl acetate (*Z*9-14:Ac) and (*Z*,*E*)-9,12-tetradecadienyl acetate (*ZE*-9,12-14:Ac). Extracts of female glands were analyzed by capillary gas chromatography on three columns of different polarities. In each analysis, peaks with retention times identical to *Z*9-14:Ac and *ZE*-9,12-14:Ac were observed. GC-MS analysis of gland extracts supported the identification of these two compounds. Volatiles emitted from female sex pheromone glands during 10-min collection periods contained 7.8 ± 2.01 ng of *Z*9-14:Ac. On average the blend contained 3.8 ± 1.43% *ZE*-9,12-14:Ac. Blends ranging from 1% to 10% *ZE*-9,12-14:Ac in *Z*9-14:Ac (1 mg) were effective in capturing males in the field. The number of males captured in traps baited with a 3% blend of *ZE*-9,12-14:Ac in *Z*9-14:Ac was not significantly different than the number caught in traps containing two virgin females.

Key Words—*Lacinipolia renigera*, Lepidoptera, Noctuidae, bristly cutworm, insect pheromones, (*Z*)-9-tetradecenyl acetate, (*Z*, *E*)-9,12-tetradecadienyl acetate, bolas spider, *Mastophora hutchinsoni*.

INTRODUCTION

The bristly cutworm, *Lacinipolia renigera* (Stephens), is a common noctuid moth in the eastern United States and has a wide host range (Rings and Arnold, 1975). In most years, this species is not considered a pest, but heavy damage to corn and feeding on alfalfa, clover, grasses, and tobacco have been reported (Knutson, 1944; Rings and Arnold, 1975). The bristly cutworm has two generations per year in Kentucky and throughout most of its range (Crumb, 1929; Garman, 1895; Rings and Arnold, 1975).

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The bristly cutworm is one of the principal prey items of a bolas spider, *Mastophora hutchinsoni* Gertsch (Yeargan, 1988), which, like other bolas spiders, hunts by releasing an attractant that mimics the sex pheromone emitted by adult female moths (Stowe et al., 1987). Based on field screening, (*Z*)-9-tetradecenyl acetate (*Z*9–14 : Ac) has been reported as a sex attractant for *L. renigera*, but the potency of lures containing this compound was rated as "low" when compared to other population indicators (Steck et al., 1982). In the present study, which was undertaken as part of a broader study aimed at gaining a better understanding of the semiochemical relationship between *M. hutchinsoni* and its lepidopterous prey, *Z*9–14 : Ac and (*Z*,*E*)-9,12-tetradecadienyl acetate (*ZE*-9,12–14 : Ac) were identified from gland extracts of female moths. A blend of these compounds, approximating the blend ratio found in female glands, proved to be as effective as females in trapping males in sticky traps.

METHODS AND MATERIALS

Adult female bristly cutworms were collected at a blacklight trap in a residential backyard in Lexington, Kentucky, during June and July 1989. After collection, the moths were placed in 1-pint (473 ml) paper cartons, which had nylon screen lids. The females oviposited on these lids. At hatching, larvae were transferred individually to 30-ml plastic cups that contained a pinto bean diet (Shorey and Hale, 1965). Larvae were periodically transferred to new cups of diet as their food desiccated. At the prepupal stage, they were transferred to a 1 : 1 soil–vermiculite mix, in which they pupated. Pupae were separated according to sex. Female pupae were housed in 1-pint paper cartons with nylon-screen lids under a 14 : 10 light–dark photoregime with diurnal temperature of 28°C and nocturnal temperature of 18°C. Three-to 5-day-old females were used in experiments to identify pheromone components.

Extracts of pheromone glands were made between the first and fourth hour of the scotophase, because males were caught by *M. hutchinsoni* only during the early part of a night (Yeargan, 1988). The ovipositor and the associated sex pheromone gland were forcibly extruded and removed with a pair of dissecting scissors. Two types of gland extracts were prepared: (1) one to three glands left in hexane for 5 min, and (2) pooled extracts containing three to nine glands left in methylene chloride for 24 hr.

Airborne collections of volatiles from forcibly extruded pheromone glands were conducted following the procedures outlined by Haynes and Hunt (1990) as modified from those developed by Baker et al. (1981). After a 10-min collection period, an internal standard [10 ng of (*E*,*Z*)-4,7-tridecadienyl acetate] was added to the glass wool at the distal end of the collector before it was rinsed with ca. 100 μl of CS_2. This volume was reduced to ca. 1 μl before gas chromatographic analysis.

Gas chromatographic analysis of gland extracts was conducted on three different types of capillary columns installed in Hewlett-Packard 5890A or Series II gas chromatographs. A 30-m DB-5 column (0.25 mm ID, J&W Scientific) was run using the following conditions: initial temperature 80°C for 2 min; temperature program 10°C/min to 220°C; He carrier gas flow at 1 ml/min. A 25-m HP-17 column (0.2 mm ID, Hewlett-Packard) was used with the same temperature program, but with maximum temperature at 210°C. A flame ionization detector was used with these columns with N_2 (makeup gas) flow at 30 ml/min, air flow at 400 ml/min, and H_2 flow at 30 ml/min. A 30-m Carbowax 20 M column (0.25 mm ID; J&W Scientific) was used in conjunction with a Hewlett-Packard 5970B mass selective detector (MSD). The temperature program for the GC was identical to that used with the HP-17 column. Initially the MSD was used in the scan mode for pooled gland extracts (scanning ions from 40 to 500 *m/z*), but for increased sensitivity single-gland extract analyses and volatile collections were analyzed using selected ion detection (*m/z* ions 54, 55, 61, 67, 166, 192, 194, 220, and 222).

For two pooled sex pheromone gland extracts (three and five female equivalents), the procedure of Buser et al. (1983) was used to determine the double-bond position in one of the putative pheromone components. After the dimethyl disulfide adducts were prepared, they were analyzed using a 30-m Carbowax 20 M column and mass spectrometry. The temperature program was modified from that listed above in that the column was held at 120°C for 2 min, and then the temperature was increased by 20°C/min to 250°C. The MSD was operated in the scan mode detecting ions from 40 to 350 *m/z*. Mass spectra and retention times were compared to those found in the dimethyl disulfide adducts of authentic *Z*9–14 : Ac.

Synthetic *Z*9–14 : Ac, *E*9–14 : Ac, *ZE*-9, 12–14 : Ac, *EE*-9,12–14 : Ac, *ZZ*-9,12–14 : Ac, *EZ*-9, 12–14:Ac, and *EZ*-4,7–13 : AC were obtained from the Institute of Pesticide Research, Wageningen, The Netherlands. Isomeric and overall purity was greater than 99% (Simon Voerman, Research Chemist, Institute for Pesticide Research). These compounds were diluted in CH_2Cl_2 before GC analysis or field experiments.

Field tests were conducted during September 1989, in a residential backyard in Lexington, Kentucky. In the first experiment, Pherocon 1C sticky traps were baited with one of four treatments, which included: (1) a 6-cm-diameter screen cage containing two 3- to 6-day-old female *L. renigera*; (2) 1 mg of *Z*9–14 : Ac on a rubber septum; (3) 1 mg of *Z*9–14 : Ac with 30 μg of *ZE*-9,12–14 : Ac on a rubber septum; and (4) a rubber septum treated with 100 μl of CH_2Cl_2 (the same volume of solvent as used with treatments 2 and 3). Rubber septa (A.H. Thomas) were extracted first with hexane and methylene chloride before synthetic compounds were added in methylene chloride. Traps were placed so that their openings were ca. 20 cm above ground with a minimum of

10 m between traps. The experimental design was a randomized complete block design, with three blocks of the experiment conducted per night for seven nights. Experimental blocks were not included in data analysis if no moths were captured with any treatment.

In a second experiment, the responses of males to the following treatments were compared: (1) 30 μg of *ZE*-9,12–14 : Ac; (2) 1 mg of Z9–14 : Ac with 10 μg (1%) of *ZE*-9,12–14 : Ac; (3) 1 mg of Z9–14 : Ac with 30 μg (3%) of *ZE*-9,12–14 : Ac; and (4) 1 mg of Z9–14 : Ac with 100 μg (10%) of *ZE*-9,12–14 : Ac. The experimental design was identical to the first experiment, except the experiment was continued for 10 nights. The number of male bristly cutworms captured during this second experiment was much lower than in the first experiment, probably because it was conducted near the end of the bristly cutworm's fall generation. Therefore, captures of males in the three replicates of each treatment per night were totaled before the statistical analysis was done. This procedure helped to control the large intertrap variation on each night. For both experiments analysis of variance was performed on square-root transformed data ($\sqrt{x + 0.5}$). Means were separated using the Scheffé method (Sokal and Rohlf, 1981). Specimens of *L. renigera* have been placed in a reference collection housed in the Department of Entomology, University of Kentucky, Lexington.

RESULTS AND DISCUSSION

Pheromone gland extracts contained compounds that had retention times identical to those of Z9–14 : Ac and *ZE*-9,12–14 : Ac when analyzed by capillary gas chromatography using three columns of differing polarity (DB-5, HP-17, and Carbowax 20 M). The analysis on the Carbowax 20 M column showed that authentic *ZE*-9,12–14 : Ac could be distinguished from *ZZ*-, *EZ*-, and *EE*-9,12–14 : Ac based on retention time. On the same column *E*- and Z9–14 : Ac could be easily distinguished.

The mass spectra of the two putative pheromone components matched those of authentic Z9–14 : Ac and *ZE*-9,12–14 : Ac. In particular, the ion at *m/z* 61 (characteristic of acetates), was detected with both compounds, the ion at *m/z* 192 (corresponding to M^+-CH_3COOH for a tetradecadienyl acetate) was associated with the compound with the retention time identical to authentic *ZE*-9,12–14 : Ac, and the ion at *m/z* 194 was detected in the compound with a retention time identical to Z9–14 : Ac.

Preparation of the dimethyl disulfide derivatives of sex pheromone gland extracts yielded a peak with a retention time and mass spectrum that matched that of dimethyl disulfide derivative of Z9–14 : Ac. In particular, ions at *m/z* 117, 171, 231, and 348 showed relative abundances that were similar in derivatives from the pheromone extract and derivatives of authentic Z9–14 : Ac. This

finding provided strong evidence that one of the unknowns was a tetradecenyl acetate with the double bond at the 9 position. These results, when considered together with the capability of the capillary Carbowax 20 M column to resolve *E* and *Z* isomers, provided evidence that one of the unknowns was Z9–14 : Ac.

The emission rate and blend ratio of the two putative pheromone components were quantified by collecting volatiles emitted from forcibly extruded sex pheromone glands. The mean emission rate of Z9–14 : Ac from three females was 7.8 ± 2.01 (SEM) ng/10 min. *ZE*-9,12–14 : Ac was present at 3.8 ± 1.43% (SEM) of Z9–14 : Ac.

In the first field experiment, traps baited with 1 mg Z9–14 : Ac on a rubber septum and control traps captured no male bristly cutworm moths. However, when 30 μg of *ZE*-9,12–14 : Ac was included with the 1 mg of Z9–14 : Ac significantly more male bristly cutworms were captured ($P < 0.05$; two-way analysis of variance, means separated by Scheffé's multiple comparison procedure) (Table 1). In fact, the number of males captured in these traps was not significantly different from the number of males captured in traps baited with two virgin females ($P < 0.05$). These results do not necessarily conflict with those of Steck et al. (1982), who found that male bristly cutworm moths were captured in traps baited with Z9–14 : Ac alone, because they found that the effectiveness of this single compound in sampling males from populations of this species was relatively low when compared to other methods of assessing the population size.

In the second field experiment, no significant differences was found in the number of males captured in traps among blends of Z9–14 : Ac (1 mg) and 10,

TABLE 1. CAPTURES OF MALE BRISTLY CUTWORM MOTHS IN PHEROCON 1C TRAPS

Traps baited with	Experiment I (mean No. of males per trap per night ± SEM, $N = 20$)[a]	Experiment II (mean No. of males per 3 traps per night ± SEM, $N = 10$)
Two virgin females	5.65 ± 1.73a	
Z9–14 : Ac (1 mg)	0b	
Z9–14 : Ac (1 mg) + 10 μg *ZE*-9,12–14 : Ac		3.00 ± 1.05a
Z9–14 : Ac (1 mg) + 30 μg *ZE*-9,12–14 : Ac	3.55 ± 0.87a	4.40 ± 0.72a
Z9–14 : Ac (1 mg) + 100 μg *ZE*-9,12–14 : Ac		2.20 ± 0.79a
30 μg *ZE*-9,12–14 : Ac		0b
Blank	0b	

[a] Means in the same column are significantly different if they do not share a letter in common ($P < 0.05$; square-root transformed data; two-way analysis of variance; Scheffé multiple comparison procedure).

30, or 100 μg of *ZE*-9,12–14:Ac (ranging from 1% to 10% relative to Z9–14:Ac) (Table 1). *ZE*-9,12–14:Ac (30 μg) by itself captured no bristly cutworms, which rules out the possibility that the response in all cases was due to *ZE*-9,12–14:Ac alone.

Z9–14:Ac is reported as a sex pheromone or sex attractant component for species of moths in the families of Gelechidae, Noctuidae, Oecophoridae, Pyralidae, Tineidae, Tortricidae, Yponomeutidae, and Zygaenidae (Arn et al., 1986, Steck et al., 1982). Demonstration of attraction of males to *ZE*-9,12–14:Ac or blends containing this compound have been limited to Noctuidae, Pyralidae, and Tortricidae (Arn et al., 1986). Within the Noctuidae, identification of *ZE*-9,12–14:Ac from female sex pheromone glands and demonstration that this compound plays a role in mate location behavior has been limited to the Amphipyrinae subfamily of Noctuidae (*Spodoptera* spp.). While blends containing *ZE*-9,12–14:Ac have been found to lead to captures of males in the subfamily Hadeninae, to which *L. renigera* belongs, there has been no previous report of identification of a sex pheromone in this subfamily that contains this compound (Arn et al., 1986).

The bolas spider, *Mastophora hutchinsoni*, releases a volatile chemical or chemicals that attract several species of moths, including *L. renigera* (Yeargan, 1988). *L. renigera* and the smoky tetanolita, *Tetanolita mynesalis* (Walker), were the most abundant prey species captured by this spider. Because *L. renigera* is caught by the spider before 11:00 PM and *T. mynesalis* is captured primarily after 11 PM, it is possible that the two species share the same pheromone components and that potentially maladaptive communicative interactions between the two species do not occur because of these temporal differences (Yeargan, 1988). However, the sex pheromone blend that was identified for *L. renigera* does not appear to be an effective attractant for *T. mynesalis*. Over the course of the two experiments, only one *T. mynesalis* was captured in traps baited with Z9–14:Ac and 3% *ZE*-9,12–14:Ac. This lack of response of *T. mynesalis* is probably not due to lack of a population from which to sample. At a location approximately 1.6 km from the field site, bolas spiders were catching both *L. renigera* and *T. mynesalis* over the course of both experiments. In addition, 22 male *T. mynesalis* were captured using a blacklight trap on a night when no male *T. mynesalis* were captured in sticky traps baited with Z9–14:Ac and 3% *ZE*-9,12–14:Ac at the same location. In the future, elucidation of the chemical composition of the attractant produced by the spider and the pheromone blends produced by each of moth species upon which it preys should help to resolve the semiochemical bases of these predator–prey interactions.

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NOVEL INTERNALLY BRANCHED, INTERNAL ALKENES AS MAJOR COMPONENTS OF THE CUTICULAR HYDROCARBONS OF THE PRIMITIVE AUSTRALIAN ANT *Nothomyrmecia macrops* CLARK (HYMENOPTERA: FORMICIDAE)

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Abstract—Internally mono- and dimethyl branched, internal alkenes, which constitute most of the cuticular hydrocarbon present on workers of the primitive Australian ant *Nothomyrmecia macrops* Clark, have been identified by gas chromatography–mass spectrometry. They are the first such alkenes reported from insects. Also present are alkanes with similar carbon skeletons. The hydrocarbon patterns of three separate *Nothomyrmecia* colonies were very similar.

Key Words—Internally branched alkenes, internal alkenes, cuticular hydrocarbons, *Nothomyrmecia macrops*, Hymenoptera, Formicidae, ants, gas chromatography, mass spectrometry.

INTRODUCTION

Hydrocarbons are major components of the lipid layers that form a protective barrier and reduce desiccation from the cuticle of most, if not all, insects. Cu-

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ticular hydrocarbon mixtures differ greatly in chemical nature between species. This variability has been studied widely and was reviewed recently by Lockey (1988) and Blomquist et al. (1987). A number of ant species and subfamilies have been examined, including *Hypoponera eduardi* (Forel) (Ponerinae) (Lange et al., 1989); *Solenopsis richteri* Forel and *S. invicta* Buren (Lok et al., 1975; Nelson et al., 1980); *Atta colombica* Guérin, *A. sexdens* (Linnaeus), and *A. cephalotes* (Linnaeus) (Martin and MacConnell, 1970) (all subfamily Myrmicinae); *Iridomyrmex humilis* (Mayr), *I. nitidiceps* André, and *I. purpureus* (F. Smith) (Dolichoderinae) (Brophy et al., 1983); *Cataglyphis cursor* (Fonscolombe) (Lange et al., 1989); *Camponotus vagus* (Scopoli) (Bonavita-Cougourdan et al., 1988; Lange et al., 1989) (both Formicinae); *Myrmecia gulosa* (Fabricius) (Myrmeciinae) (Cavill et al., 1970), and *Pseudomyrmex ferrugineus* (F. Smith) (Pseudomyrmicinae) (Mintzer et al., 1987).

Insect cuticular hydrocarbons fall largely into three classes; *n*-alkanes, methyl-branched alkanes, and *n*-alkenes. Branched alkenes appear to be very rare; the only records known to us are of terminally branched tetra-, penta-, and hexatriacontenes from the house cricket, *Acheta domesticus* (Linnaeus) (Warthen and Uebel, 1980; Anderssohn and Hadley, 1987), and 13-methyl-1-hentriacontene and 13-methyl-1-tritriacontene (which function as sex pheromones) from the stable fly, *Stomoxys calcitrans* Linnaeus (Sonnet et al., 1979). Internally branched, internal alkenes have not been recorded previously from insects.

The rare Australian "dinosaur ant," *Nothomyrmecia macrops* Clark, is arguably the most primitive extant formicid and is believed to be a "living fossil" representing a very ancient lineage in ant evolution. It is classified separately from all other ants in the monotypic subfamily Nothomyrmeciinae (Clark, 1951; Taylor, 1978). In this paper we report internally branched, internal alkenes as the major constituents of the cuticular hydrocarbons of *N. macrops* workers.

METHODS AND MATERIALS

Ants. Five to 10 *N. macrops* workers were collected in November 1988 at Poochera, South Australia, from each of three colonies. Specimens of queens and males were not available for this study; whole colonies would be destroyed to obtain the former, and the latter are highly seasonal and seldom accessible. Because *Nothomyrmecia* is an exceptionally rare ant, known only from an area of about 1 km^2 surrounding Poochera, the number of colonies sampled and the number of workers taken from each were limited.

Chemical Methods. Cuticular hydrocarbons were removed by immersing

each insect for 10 min in three successive portions of hexane. The combined samples were placed on 5-cm columns of Bio-Sil A (Bio-Rad Lab., Richmond, California) in Pasteur pipets, eluted with hexane, and concentrated under nitrogen. Samples were fractionated subsequently on a silver nitrate treated Bio-Sil column; saturates were eluted with hexane and unsaturates with ether.

Catalytic reduction was carried out by bubbling hydrogen through an ethanolic solution of the alkenes in a Reacti-vial, with a trace of Adams platinic oxide catalyst.

Gas Chromatography. Gas chromatography (GC) was performed with a Varian 1400 gas chromatograph equipped with a splitless injector and a 12-m × 0.53-mm methylsilicone column (S.G.E., BP1, phase thickness 1 μm). The injector and flame ionization detectors were held at 310°C, and the oven programmed from 200°C to 310° at 4°/min. Helium with a flow rate of 4 ml/min was the carrier gas. Percentage compositions were obtained using a Hewlett-Packard 3390A integrator. Alternatively, a Varian 3300 gas chromatograph with an ambient on-column injector and a 30-m × 0.32-mm methylsilicone column (J&W Scientific, DB-1, phase thickness 0.25 μm) was used. Injection was made at 60°C, followed by a rapid rise at 50°/min to 220°C, then programming at 4°/min to 310°C; the detector was held at 320°C. Helium with a flow rate of 1.5 ml/min was the carrier gas. Data were processed using an Epson PCe computer with an A to D converter and Data Acquisition, Plotting and Analysis software (Sheppard and van Bronswijk, 1987). The equivalent chain lengths (ECLs) of components were calculated by comparison of retention times with those of *n*-alkane standards.

Gas Chromatography–Mass Spectrometry. Gas chromatography–mass spectrometry (GC-MS) was carried out on a VG 70-70 mass spectrometer coupled directly to a Hewlett-Packard 5792A gas chromatograph and a VG 11-250 data system. Injections were made on-column at 60°C. The 5% phenylmethylsilicone columns (S.G.E., BP-5, 25 m × 0.33 mm ID, phase thickness 0.5 μm) with helium or hydrogen flow of 1 ml/min were used with appropriate temperature programming to 315°C to allow expeditious elution of components. Mass spectra were obtained using electron ionization (EI, 70 eV) at a source temperature of 200°C, an accelerating voltage of 4 kV, and a scanning rate of 0.7 Hz.

Component hydrocarbons were identified by well-established procedures using GC-MS (Blomquist et al., 1987; Lockey, 1988). Molecular weights and branching positions in alkanes were determined from the mass spectra, which showed enhanced ions corresponding with alpha cleavage at the branching point, in conjunction with ECLs. Double bond locations were determined by GC-MS analyses of dimethyl disulfide (DMDS) derivatives (Dunkelblum et al., 1985) in conjunction with microscale ozonolysis. Branching positions in the alkenes were established after catalytic reduction to the corresponding alkanes.

RESULTS

The GC profiles of the three subject worker series of *N. macrops* are shown in Figure 1. They evidence very similar hydrocarbon compositions, with only minor quantitative variation between the samples. Because of these similarities, and of the limited material available, the samples were pooled for subsequent chemical analysis.

Saturated alkanes constituted approximately 35% of total hydrocarbons, and the results of analysis are presented in Table 1. Monomethyl internally branched alkanes (i-methylalkanes) (55%) were the major hydrocarbon type present, with most of the remainder being dimethylalkanes, while 2- and 3-methylalkanes were poorly represented. A small quantity only of a single *n*-alkane (nonacosane) was detected (other *n*-alkanes, if present, would have been detected by GC and GC-MS, despite adjacent dimethylalkanes). The mass spectra showed that 7-methylalkanes were the major components of the i-methylalkanes of odd chain length and that the dominant chain length was 31. i-Methylalkanes of even chain length had branching almost entirely on even-numbered carbon atoms.

GC analysis of the unsaturated fraction (ca. 65% of total hydrocarbons) indicated that these compounds were unusual. Cuticular alkenes are normally straight chain, with one, two, or occasionally three double bonds, and with odd chain lengths predominant. As the degree of unsaturation progressively reduces the ECL below that of the corresponding *n*-alkane, the major cuticular alkenes normally have ECLs of ca. $x.4$ to $x.8$ (where x is an even integer), although some trienes can have ECLs of ca. $x.2$. It was clear that the major alkenes of *N. macrops* (see Table 4 below) did not fit this pattern. These compounds, therefore, must have been highly unsaturated or branched. The molecular weights (derived from MS) showed that, in fact, they were all monoenes; implying that the unsaturated hydrocarbon fraction of *N. macrops* contained a series of branched alkenes.

This was confirmed when the unsaturated fraction was subjected to catalytic reduction followed by GC-MS analysis to reveal a series of monomethyl- and dimethyl-alkanes (Table 2). These alkanes had structures similar to those of the saturated fraction, except that the predominant chain length was now 33 (68%), not 31.

The unsaturated fraction was treated with dimethyl disulfide and the resulting DMDS derivatives analyzed by GC-MS (for example Figure 2B). This procedure identifies the molecular weight of each parent alkene and the size of the alkyl substituents of its double bond. However, the EI mass spectra of the DMDS derivatives do not display distinctive features indicating which of the two alkyl substituents contains the branching methyl groups.

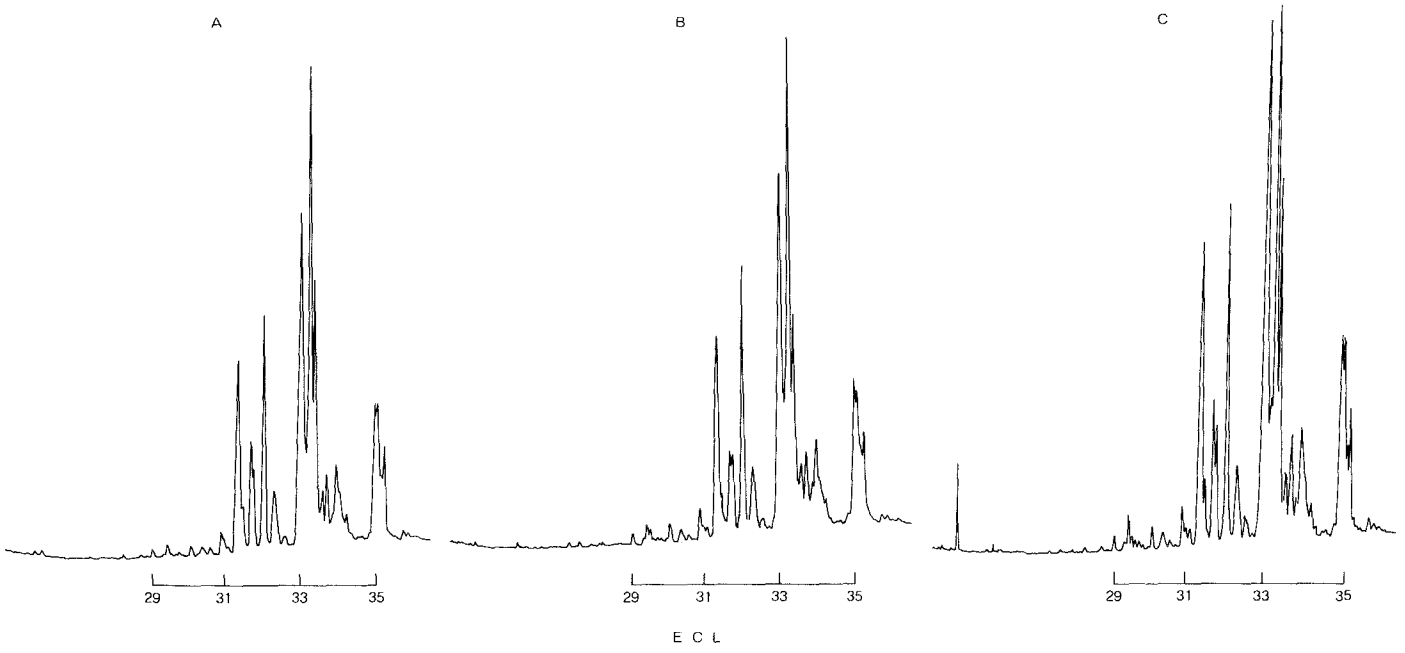


FIG. 1. Gas chromatograms of cuticular hydrocarbons of three colonies of *Nothomyrmecia macrops*. The scale line is equivalent chain length (ECL). The column used was a 30-m × 0.32-mm methylsilicone (J&W, DB-1, phase thickness 0.25 μm). Injection was at 60°C followed by a rapid temperature rise to 220°C then programming at 4°/min. to 310°C.

TABLE 1. SATURATED CUTICULAR HYDROCARBONS OF *Nothomyrmecia macrops*

ECL[a]	Percent[b]	Compound[c]	Diagnostic ions *m/z*[d]
27.35	0.2	13-; 11-MeC27	196/197, 224/225; 168/169, 252/253
28.63	0.8	unknown	
29.00	1.4	*n*-C29	408
29.40	3.0	15-; 13-; 11-; 9-; 7-(m); 5-(tr)C29	224/225 (sym), 196/197, 252/[253]; 168/169, [280]/281; 140/141, 308/309; 112/113, 336/337; 84/85, [364]/365
29.73	0.3	3-MeC29	393
30.09	1.3	3,9-; 3,7-diMeC29	154/155, 308/309, 407; 126/127, 336/337, 407
30.39	1.9	10-; 8-(m); 7-; 6-MeC30	154/155, 308/309; 126/127, [336]/337; 112/113, 350/351; 98/99, 364/365
30.64	0.2	2-(or 4-)MeC30 (pr)	393, [421]
30.85	3.6	3-MeC30 (pr); 4(or 2), 10-; 4(or 2), 8-diMeC30	407; 168/169, 308/309, 407; 140/141; 336/337, 407
31.39	27.3	11-; 9-; 7-(m); 5-MeC31	168/169, 308/309; 140/141, 336/337; 112/113, 364/365; 84/85, 392/393
31.76	13.5	3-MeC31; 5,11-; 5,9-diMeC31	421; 84/85, 182/183, 308/309, 407; 84/85, 154/155, 336/337, 407
32.06	19.1	3,11-; 3,9-; 3,7-diMeC31	182/183, 308/309, 435; 154/155, 336/337, 435; 126/127, 364/365, 435.
32.40	6.5	12-; 10-; 8-; 7-(tr); 6-MeC32	182/183, 308/309; 154/155, 336/337; 126/127, 364/365; 112/113, 378/379; 98/99, 392/393
32.65	0.7	2-(or 4-)MeC32 + unknown?	420/421, 449
32.90	1.2	4(or 2), 10-; 4(or 2), 8-diMeC32	168/169, 336/337, 435, 463; 140/141, 364/365, 435, 463
33.42	15.6	13-; 11-; 9-; 7-(m)MeC33	196/197, 308/309; 168/169, 336/337; 140/141, 364/365; 112/113, 392/393
33.61	1.2	11,21-; 7,11-diMeC33 (pr)	168/169, 196/197, 322/323, 350/351; 112/113, 182/183, 336/337, 406/407
34.07	1.5	3,11-; 3,9-(m); 3,7-diMeC33	182/183, 336/337, 463; 154/155, 364/365, 463; 126/127, 392/393, 463
34.55	0.9	12-; 10-; 8-MeC34	178/[179], 336/337; 154/155, 364/365; 126/127, [392]/393

[a] Equivalent chain length.
[b] Percentage of total saturates.
[c] 11-MeC27 denotes 11-methylheptacosane; (m) signifies mainly, (tr) trace, and (pr) a provisional identification based on ECL and/or weak, incomplete or difficult to resolve mass spectra. 2- and 4-methylalkanes have very similar mass spectra but the branching position given first is the more likely.
[d] Diagnostic ions indicating branching points, ions in brackets were not detected.

TABLE 2. REDUCED UNSATURATED CUTICULAR HYDROCARBONS OF *Nothomyrmecia macrops*

ECL[a]	Percent[b]	Compound[c]	Diagnostic ions *m/z*[d]
23.00	1.1	*n*-C23[e]	324
25.00	0.2	*n*-C25[e]	352
33.40	54.0	11-; 9-; 7-(m); 5(tr)-MeC33	168/169, 336/337; 140/141, 364/365; 112/113, 392/393; 84/85, 420/421
33.78	7.1	3-MeC33; 5,9-diMeC33	448/449; 84/85, 154/155, 364/365, 434/435
34.11	7.1	3,9-; 3,7-diMeC33	154/155, 364/365, 463; 126/127, 397/398, 463
34.42	6.6	10-; 8-; 6-MeC34	154/155, 364/365; 126/127, 392/393; 98/99, 420/421
35.38	20.6	11-; 9-; 7-(m)MeC35	168/169, 364/365; 140/141, 392/393; 112/113, 420/421
36.07	1.1	3,11-; 3,9-; 3,7-diMeC35	182/183, 364/365, 491; 154/155, 392/393, 491; 126/127, 420/421, 491
36.32	0.6	10-; 8-MeC36	154/155, 392/393; 126/127, [420]/421
37.30	1.0	11-; 9-; 7-(m)MeC37	168/169, 392/393; 140/141, 420/421; 112/113, 448/449

[a] Equivalent chain length.
[b] Percentage of total reduced alkenes.
[c] 11-MeC33 denotes 11-methyltritriacontane; (m) signifies mainly, and (tr) trace.
[d] Diagnostic ions indicating branching points, ions in brackets were not detected.
[e] Probably derived from Dufour gland.

The double bonds were located unambiguously with respect to the branching methyl groups by the independent method of ozonolysis of the unsaturated fraction. GC analysis of the product, in comparison with standard *n*-aldehydes, showed a series of even-chain-length *n*-aldehydes with chain lengths of 6–16 [except for a little *n*-nonanal derived from 9-tricosene, which is probably a Dufour gland component (Attygalle and Morgan, 1984; Billen et al., 1988; Lanne et al., 1988) rather than a cuticular hydrocarbon]. *n*-Dodecanal (45%) and *n*-tetradecanal (30%) were predominant. These data showed unequivocally that the smaller of the two alkyl substituents of each double bond was exclusively straight chain. The presence of the branching methyl groups in the larger alkyl substituents was reflected by a complex mixture of presumably methyl branched aldehydes of longer chain length, which eluted at longer retention times. Shortage of material and lack of suitable standards prevented further analysis of these compounds.

The double-bond locations of the constituents of the unsaturated fraction are summarized in Table 3. The double bonds were located almost exclusively

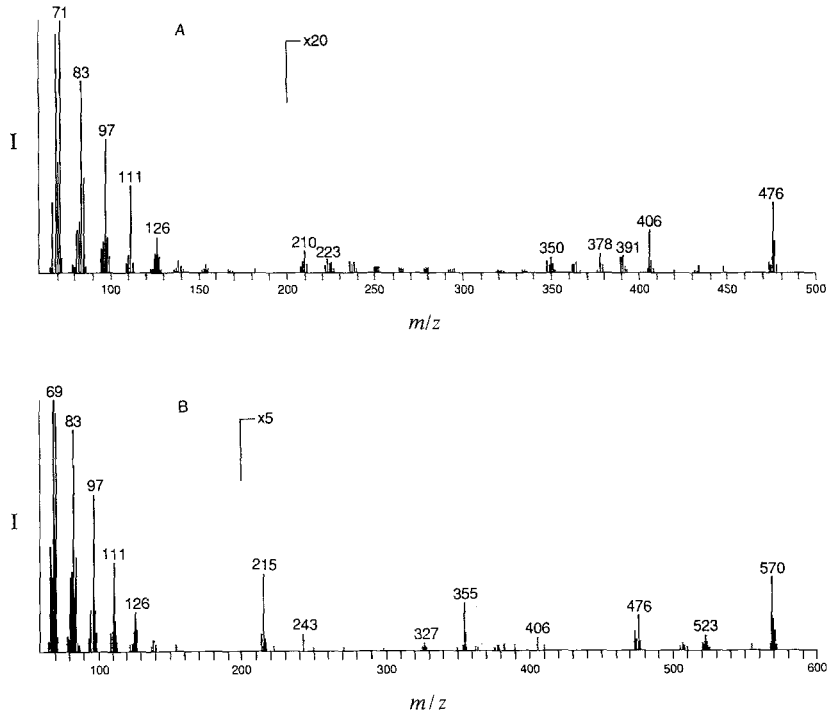


FIG. 2. (A) Electron ionization mass spectrum of a methylalkene mixture (mainly 27-methyltritriacontenes, ECL ca. 33.07). (B) Electron ionization mass spectrum of a mixture of DMDS adducts (mainly those of 27-methyl-12-tritriacontene and 27-methyl-14-tritriacontene).

at even positions (Δ^6 to Δ^{16}), with those at Δ^{12} and Δ^{14} most abundant. Even the normal alkene 9-tricosene has its double bond 14-carbon atoms from the other terminus.

An EI mass spectrum representing mainly the DMDS derivative of 27-methyl-12-tritriacontene (Δ^{12}-27-methyltritriacontene) is illustrated in Figure 2B. This shows the molecular ion (*m/z* 570) and major daughter ions (*m/z* 215 and 355). The less abundant daughter ions at *m/z* 243 and 327 represent a smaller quantity of the isomeric 27-methyl-14-tritriacontene. Also evident in the mass spectrum is dimethyldisulfide elimination to give the original alkene(s) (*m/z* 476) together with its characteristic daughter ions (*m/z* 126 and 406) (Carlson et al., 1989).

In contrast to the branched alkanes, the favored bond cleavage in the EI mass spectra of the branched alkenes was *beta* to the branching point, rather than *alpha*. The unexpected change in reaction pathway appears to arise from rapid isomerization of the molecular ions of the branched alkenes. Studies of

TABLE 3. DOUBLE-BOND POSITIONS FOR *Nothomyrmecia macrops* UNSATURATED CUTICULAR HYDROCARBONS AS DETERMINED FROM MASS SPECTRA OF DMDS DERIVATIVES

ECL[a]	Double-bond position[b]	Diagnostic ions *m/z*[c]
22.75	Δ^9-C23 : 1[d]	173, 243, 416
33.07 33.30	Δ^6-; Δ^8-(tr); Δ^{10}-(s); Δ^{12}-(m); Δ^{14}-(s); Δ^{16}-(tr)MeC33 : 1	131, 439, 570; 159, 411, 570; 167, 383, 570; 215, 355, 570; 243, 327, 570; 271, 299, 570
33.61	Δ^{12}-; Δ^{14}-diMeC33 : 1	215, 369, 584; 243, 341, 584
33.74	Δ^{10}-; Δ^{12}-diMeC33 : 1	187, 369, 584; 215, 341, 584
34.00	Δ^6-; Δ^8-(tr); Δ^{10}-(s); Δ^{12}-; Δ^{14}-MeC34 : 1	131, 453, 584; [159], 425, 584; 187, 397, 584; 215, 369, 584; 243, 341, 584
35.02	Δ^8-; Δ^{14}-MeC35 : 1	159, 439, 598; 243, 355, 598
35.75	Δ^{14}-diMeC35 : 1	243, 369, [612]

[a] Equivalent chain length of underivatized alkene.
[b] Δ^{12}-MeC34 : 1 denotes an alkene with a 34-carbon chain, with a double bond 12 positions from the nearer end of the chain and a branching methyl group; (m) signifies mainly, (s) small, and (tr) trace.
[c] Diagnostic ions showing molecular weight and point of schism of the DMDS derivative, ions in brackets were not detected.
[d] Probably derived from Dufour gland.

the mass spectra of isomeric octenes at very short ion lifetimes have established that loss of positional integrity of the double bond, to give a mixture of interconverting molecular ion structures, occurs within 10^{-9} sec (Borchers et al., 1977b). The mechanism for isomerization involves fast hydrogen rearrangements rather than cyclizations (Borchers et al., 1977a) or other skeletal rearrangements (Levsen, 1975). An analogous isomerization process preceding fragmentation for molecular ions of the branched alkenes would lead to delocalization of the double bonds, but with preservation of the positions of branching. For example, with molecular ions of the predominant 27-methyl-12-tritriacontene (Δ^{12}-27-methyltritriacontene, $M^+ \cdot$ *m/z* 476, Figure 2A), migration of the unsaturation to the Δ^{30} position would facilitate ion fragmentation through rearrangement of the 27-methine hydrogen atom to the double bond, *via* a favored six-centered transition state with bond cleavage *beta* to the branching point, yielding the daughter ion of *m/z* 406. The complementary hydrogen rearrangement and *beta* cleavage would occur after migration of the double bond to the Δ^{23} position, yielding the daughter ion of *m/z* 126.

The branched alkenes identified in *N. macrops* are summarized in Table 4, which also indicates the diagnostic MS ions that confirm the branching posi-

TABLE 4. UNSATURATED CUTICULAR HYDROCARBONS OF *Nothomyrmecia macrops*

ECL[a]	Percent[b]	Compound[c]	Diagnostic ions *m/z*[d]
22.75	1.0	Δ^9-C23 : 1	322
24.64	0.2	C25 : 1	
33.07	31.4	Δ^6-/Δ^8-(tr)/Δ^{10}-(s)/Δ^{12}-(m)/Δ^{14}-/	182, 350, 476; 154, 378, 476;
33.30	25.1	Δ^{16}-(tr)23-; 25-; 27-(m); 29-(tr)	126, 406, 476;
		MeC33 : 1	*
33.61	3.9	Δ^6-/Δ^8-(tr)/Δ^{10}-(s)/Δ^{12}-(m)/Δ^{14}-(s)/	*
		Δ^{16}-(tr)3-MeC33 : 1	
		Δ^{12}-/Δ^{14}-25,29-diMeC33:1	168, 378, 490
33.74	3.8	Δ^{10}-/Δ^{12}-25,31-; 27,31-diMeC33 : 1	168, 378, 490, 140, 406, 490
34.00	7.6	Δ^6-/Δ^8-(tr)/Δ^{10}-(s)/Δ^{12}-/Δ^{14}-25-;	168, 378, 490, 140, 406, 490;
		27-; 29-MeC34 : 1	112, 434, 490
34.25	1.6	unknown	
34.52	0.4	unknown	
34.68	0.4	unknown	
35.02	17.1	Δ^8-/Δ^{14}-25-; 27-; 29-(m)MeC35 : 1	182, 378, 504; 154, 406, 504;
35.16	5.4		126, 434, 504
35.74	1.0	Δ^{14}-25,33-; 27,33-; 29,33-	196, 378, 518; 168, 406, 518;
		diMeC35 : 1	140, 434, 518
37.00	0.8	27-; 29-; 31-(tr)MeC37 : 1[e]	182, 406, 532; 154, 434, 532;*

[a] Equivalent chain length.

[b] Percentage of unsaturates.

[c] Δ^{12}-27-MeC33 : 1 denotes 27-methyl-12-tritriacontene; Δ^{14}-25,33-diMeC35 : 1 denotes 25,33-dimethyl-14-pentatriacontene; in most cases GC peaks do not represent single compounds but overlapping components with branching positions and double-bond positions as indicated; (m) signifies mainly, (s) small, and (tr) trace.

[d] Diagnostic ion arising from beta cleavage supporting previously determined methyl branching position (Table 2); asterisks denote components the ions of which were not detected but were evident in the reduced alkenes.

[e] The mass spectra of the DMDS derivatives of these compounds were too poor to enable double-bond location.

tions given in Table 2. In most cases the GC peaks, or groups of peaks, do not represent single components, but are rather an envelope from many overlapping peaks. Thus, for the group of peaks with ECL 33.07–33.30, although the largest single component is almost certainly Δ^{12}-27-methyltritriacontene, it is likely that compounds with many of the other possible combinations of methyl-branching positions and double-bond positions are also present. Although we have not directly assigned the geometry of the double bonds, insect cuticular alkenes normally have the *Z* configuration.

DISCUSSION

The most remarkable feature of the cuticular hydrocarbons of *N. macrops* is the presence of large quantities of internally branched, internal alkenes, which

account for ca. 65% of all hydrocarbons, and almost all of the unsaturates. As mentioned above, the only previous reports known to us of branched alkenes are from *Acheta domesticus* and *Stomoxys calcitrans*. In the former the branching is terminal, the compounds occur as fairly minor components, and these coelute with various *n*-alkenes (Warthen and Uebel, 1980; Anderssohn and Hadley, 1987). Methyl-branched alkenes comprise ca. 50% of the total unsaturates of *S. calcitrans*, but in this case the double bond is terminal, even though the methyl branching is internal. These compounds occur in females and appear to function as sex pheromones, which elicit a response from courting males (Sonnet et al., 1979). Thus, the occurrence of branched unsaturates is very uncommon among insect cuticular hydrocarbons, so much so that many authors have simply reported the presence of alkenes, without specifying that these are, in fact, *normal* alkenes.

The occurrence of branched alkenes in the cuticular hydrocarbons of *N. macrops* would be unusual in itself, but the facts that these compounds are novel, being internally branched and internally unsaturated, and that they are present as major components, in the absence of any significant quantities of *n*-alkenes, make the cuticular chemistry of this ant even more noteworthy. Double-bond location at even-placed carbon atoms (from the nearer end of the chain) is unusual in monoalkenes; odd-numbered positions are rather more common (Lockey, 1988).

The saturated hydrocarbons, although predominantly of odd chain length (as is usual), nonetheless show a number of interesting features. Most obvious is the almost total lack of *n*-alkanes—the only one detected was *n*-nonacosane, and it constituted a mere 0.5% of the total hydrocarbons. The only insects for which *n*-alkanes were not reported in Lockey's (1988) review were flies of the genera *Drosophila*, *Glossina*, and *Stomoxys*. Among ants, *Hypoponera eduardi* contains only a small amount of *n*-heptacosane, and *n*-alkanes are only minor components in *Atta colombica* (Martin and MacConnell, 1970) and *Iridomyrmex nitidiceps* (Brophy et al., 1983), but this feature is not evidenced in other congeneric species.

The odd-chain-length methyl-branched alkanes follow the normal pattern in having branching exclusively on odd-numbered carbon atoms, although the predominance of substitution at the seven position is unusual. Normally where chain lengths are ca. 30 or above branching would be expected mainly at position 9 or higher. In the case of the even-chain-length homologs, methyl side chains are normally positioned at both odd- and even-numbered positions (Lockey, 1988). However, in the *N. macrops* alkanes, they are almost exclusively at even positions from the nearest terminus. The alkanes produced by catalytic reduction of the unsaturates also showed these characteristics.

Considerable work has been done on the biosynthesis of cuticular hydrocarbons (Blomquist et al., 1987; Lockey, 1988, and references therein). Most studies indicate that alkenes are biosynthesized from unsaturated acids by chain

elongation followed by decarboxylation. i-Methyl-branched alkanes are considered to be synthesized by an elongation–decarboxylation pathway, with malonyl units providing the straight-chain sections and methylmalonyl units the methyl side chains. We have no evidence concerning the biosynthetic pathways used by *N. macrops* to produce these branched alkenes, but the similarity of the carbon skeletons of the saturates to those of the unsaturates suggest that the pathways leading to these alkenes and alkanes have much in common. Our work was done using only 20–30 individuals of this rare and significant ant. If sufficient numbers could be obtained for systematic biosynthetic investigation, the results might well be informative concerning insect hydrocarbon biosynthesis in general.

Nothomyrmecia macrops is the sole member of its genus and subfamily. The closest related genus is considered to be *Myrmecia*, which includes the so-called "bulldog ants" of Australia and New Caledonia and which, like *Nothomyrmecia*, also constitutes a subfamily in ant classification (Taylor, 1978). The only published work on this genus appears to be that of Cavill et al. (1970) on *Myrmecia gulosa*, where the major hydrocarbons were an homologous series of monomethyl alkanes x-MeCy (x = 9, 11, 13, 15; y = 25, 27, 29, 31, 33), and no unsaturates were detectable. Our preliminary investigations of another bulldog ant (*Myrmecia pyriformis* F. Smith) indicated a complex array of hydrocarbons, all of which appear to be saturated.

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IN VITRO ALGAL GROWTH INHIBITION BY PHYTOTOXINS OF *Typha latifolia* L.

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Abstract—The ether extract of *Typha latifolia* L. inhibited the growth of some microalgae. Among the substances chromatographically isolated and characterized from the ether extract, three steroids [β-sitosterol, (20S) 24-methylenlophenol, and stigmast-4-ene-3,6-dione] and three fatty acids [α-linolenic, linoleic, and an unidentified $C_{18:2}$] were found to inhibit the growth of some microalgae tested. A selective effect of these substance on blue-green algae (particularly on T 625 *Synechococcus leopoliensis* and on T 1444 *Anabaena flosaquae*) was observed.

Key Words—Allelopathy, *Typha latifolia*, microalgae, unsaturated C_{18} fatty acids, β-sitosterol, (20S) 24-methylenlophenol, stigmast-4-ene-3,6-dione, flavonolglycosides, growth inhibition, algal growth, phytotoxins.

INTRODUCTION

There is increasing evidence that allelopathy is an important strategy in the biology of many weeds. This research has focused on agriculture and forestry, while less evidence is available on other aspects, such as the chemical interactions among aquatic weeds and algae (Rice, 1984).

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Hasler and Jones (1949) first observed the inhibition of phytoplankton by aquatic weeds. Subsequently, Fitzgerald (1969) indicated that some aquatic weeds cultured in nitrogen-limited media remained relatively free of epiphytic algae. Harrison and Chan (1980) showed that extracts of *Zostera marina* leaves prevented the growth of epiphytes, algae, and bacteria. Recently, Della Greca et al. (1989) found that some phenylpropanes isolated from *Acorus gramineus* inhibited the growth of some blue-green and green algae. One of the most widespread aquatic weeds is *Typha latifolia* L. (cattail), which tolerates a broad range of climatic conditions. *T. latifolia* has been found in wetlands throughout boreal, temperate, and tropical regions of the world, but it can thrive also in dry continental climates. The weed is considered noxious in irrigated agricultural lands, such as rice fields (Muenscher, 1955). Moreover, cattails invading irrigation canals can arrest the water flow, causing siltation (Grace and Harrison, 1986), and in lakes they cause significant amounts of water loss through evapotranspiration (Otis, 1914).

Allelopathic interference of cattail on other aquatic weeds has been reported (McNaughton, 1968; Szczepánska, 1971), although no data are available on the effects of *T. latifolia* on algae. This study reports the isolation and the characterization of compounds from cattail, which are active in vitro against microalgae.

METHODS AND MATERIALS

Plant Collection and Extraction. Fresh, whole plants of *T. latifolia* (Typhaceae) were collected along a ditch near the Garigliano River (Campania, Italy) in September 1988, and dried at room temperature. Dried plants (7 kg) were sequentially extracted with ethyl ether, chloroform, methanol in a Soxhlet apparatus to give, respectively, 8.5 g, 15.0 g, and 17.1 g of material after evaporation of the solvents in vacuo. All the extracts were frozen and stored at −20°C until their utilization.

Bioassay. All the algal strains were cultivated on Bold basal medium (BBM) (Nichols, 1973). The growth of each alga was followed at 550 nm with a colorimeter (Bausch & Lomb Spectronic 20). During late exponential phase (ca. 0.7 absorbance units) 1 ml of each algal culture was inoculated on a Petri dish containing 30 ml of BBM solidified with 1.5% agar. The fractionated material and the purified compounds were dissolved in acetone (80 μl/mg), and a known quantity of each solution to be tested was absorbed on a paper disk (sterile blanks, Difco Bacto Concentration Disks, 6 mm). After evaporation of the solvent, the filter papers were placed on each previously inoculated dish. A control dish containing only acetone on the paper disk also was prepared. The phytotoxicity of cattail material was compared with that of the algicide $CuSO_4$ using amounts of 0.5–1 μmol.

The plates were aseptically incubated upside down at 20°C with a total irradiation of 390 $\mu E/m^2/sec$ provided by daylight fluorescent lamps from below the plates. The photoperiod was 16 hr light–8 hr dark.

The observation for inhibition of growth of the plated algae around the filter disk was made daily as soon as growth became visible. The inhibition is given as total diameter of the zone of no growth including the paper disk. Tests with α-linolenic aacid in the presence of an antioxidant were carried out by adding aseptically 5 μmol of 1-ascorbic acid to the sterilized medium.

Ethyl Ether Extract Separation. The ether extract was redissolved in ethyl ether (500 ml) and shaken with 2 M aqueous NaOH (2 × 200 ml). The organic layer was washed with water until neutral and dried on Na_2SO_4 to give, after evaporation of the solvent in vacuo, 6.3 g of neutral material. The alkaline solutions were reacidified with 1 M aqueous H_2SO_4 and extracted with ethyl ether. The organic phase was washed with water until neutral, dried on Na_2SO_4 and evaporated in vacuo to give 2.2 g of acidic material.

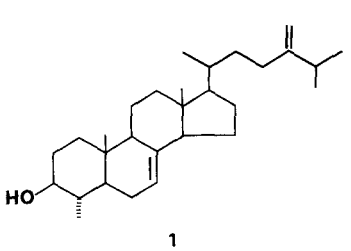
Acidic Compounds Isolation. The acidic material was chromatographed on a column of acid alumina (grade III, 22 g) using hexane containing increasing percentages of ethyl ether as eluent: the fraction eluted with a 9 : 1 mixture (62 mg; 110 ml) was chromatographed by reverse-phase HPLC on a Varian VISA 5500, equipped with a refractive index detector (Varian RI 3, Hibar LiChosorb RP-8) (5 μm; 250 × 4 mm ID, methanol–water 8 : 2) to give three acids. Aliquots of these compounds were treated with an ether solution of CH_2N_2 to give the corresponding methyl esters, which were identified as methyl α-linolenate, methyl linoleate, and an isomeric methyl linoleate. Methyl σ-linolenate had a molecular ion at *m/z* 292 in its EI-MS spectrum (Kratos MS 80 apparatus at 70 eV with a source at 250°C), corresponding to $C_{19}H_{32}O_2$, and its [^{1}H]NMR spectrum (Bruker AM 400 spectrometer in $CDCl_3$ solution) showed six olefinic protons at δ 5.42, two methylenes α to two double bonds at δ 2.79, two allylic methylenes at δ 2.08, four methylenes at δ 1.26, methylenes α and β to the carbomethoxyl group at δ 2.30 and 1.61, respectively, and finally a methyl triplet at δ 0.98, which by irradiation at δ 2.08 collapsed into a singlet. GLC (Carlo Erba Fractovap 4160) apparatus equipped with an OV-1 column (25 m × 0.32 mm ID, film thickness 0.1–0.15 μm with a column temperature of 180°C) showed identical retention time with an authentic sample purchased from Sigma Chemical Company.

Methyl linoleate had a molecular ion at *m/z* 294 corresponding to $C_{19}H_{34}O_2$; its [^{1}H] NMR spectrum showed four olefinic protons at δ 5.44, a methylene α to two double bonds at δ 2.80, two allylic methylenes at δ 2.07, six methylenes at δ 1.26, two methylenes at δ 2.30 and 1.61, and a methyl at δ 0.89. These data, as well as GLC comparison with an authentic sample (Sigma), confirmed the assigned structure.

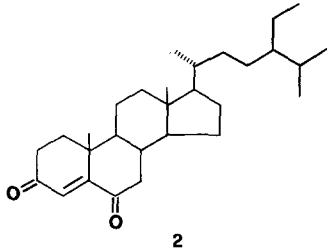
The unknown $C_{18:2}$ methyl ester showed a molecular ion at *m/z* 294 cor-

responding to $C_{19}H_{34}O_2$, and [^{1}H]NMR spectrum identical with that of methyl linoleate. The retention times in GLC and HPLC analysis were different from those of methyl linoleate.

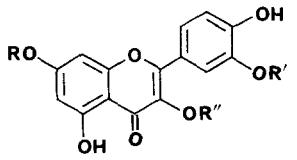
Neutral Compound Isolation. The neutral fraction (6.3 g) was chromatographed on a column of silica gel (65 g). The material (880 mg) eluted with a hexane–ethyl ether mixture (8:2; 1300 ml) was rechromatographed on silica gel using $CHCl_3$ as eluent to give mainly two fractions. The first fraction (100 mg) was purified by preparative TLC (benzene–ethyl ether 9:1; two runs) and then by C-18 reverse-phase HPLC (MeOH–H_2O, 9:1) to give (20S) 24-methylenlophenol (**1**), mp 176–178°C (Figure 1); $[\alpha]_D = +3°$ ($c = 0.8$ in $CHCl_3$). EI-MS: *m/z* 412.3681, corresponding to $C_{29}H_{48}O$, 397.3450, 328.2739, 287.2320, 285.2190, 260.2166; [^{1}H]NMR: δ 5.182 (1H, m), 4.709 and 4.662 (2H, ss), 3.131 (1H, m), 1.037 (3H, d, $J = 6.9$ Hz), 1.032 (3H, d, $J = 6.8$ Hz), 0.997 (3H, d, $J = 6.3$ Hz), 0.985 (3H, d, $J = 6.5$ Hz), 0.836 (3H, s), 0.546 (3H, s). The second fraction (84 mg) was rechromatographed on silica gel (8.4 g) and eluted with hexane–AcOEt (19:1, 100 ml) to give a mixture of β-sitosterol and stigmast-4-ene-3,6-dione (**2**), which were separated by preparative TLC (hexane–AcOEt, 8:2).



1



2



3 R = H R' = Me R'' = glu

4 R = H R' = H R'' = glu—rha

5 R = H R' = Me R'' = glu—rha

6 R = rha R' = Me R'' = glu—rha

FIG. 1. Structures **1–6.**

β-sitosterol had EI-MS *m/z* 414 corresponding to $C_{29}H_{50}O$; [^{1}H]NMR: δ 5.353 (1H, m), 3.521 (1H, m), 1.009 (3H, s), 0.921 (3H, d, J = 6.4 Hz), 0.848 (3H, t, J = 7.8 Hz), 0.832 (3H, d, J = 6.8 Hz), 0.689 (3H, s). These data were identical to those of an authentic sample (Sigma).

Compound **2** had EI-MS *m/z* 426 corresponding to $C_{29}H_{46}O_2$; UV λ_{max} (EtOH) 255 nm (log ϵ 4.05) (Perkin Elmer lambda 7 spectrophotometer). [^{1}H] NMR: δ 6.178 (1H, s), 1.169 (3H, s), 0.939 (3H, d, J = 6.5 Hz), 0.854 (3H, t, J = 6.9 Hz), 0.843 (3H, d, J = 6.1 Hz), 0.820 (3H, d, J = 6.4 Hz), 0.728 (3H, s). These data were in accord with those reported by Huizhong et al. (1985).

Fractionation of Methanolic Extract. The methanolic extract was fractionated on Amberlite XAD-2 (Rosler and Goodwin, 1983) into a saccharide fraction eluting with water and a flavanol glycosidic fraction eluting with methanol. This latter (1.7 g) was chromatograped on silica gel using $CHCl_3$–MeOH–H_2O (13 : 7 : 2; lower phase) as eluent to give nine flavonolglycosides, according to Won et al. (1983) and Ishida et al. (1988). Compounds **3**, **4**, **5**, and **6**, whose structures were attributed on the basis of a comparison of their physical features with previously reported data, were tested for their biological activity.

Compound **3** (isorhamnetin-3-O-glucoside) (21 mg) had [^{1}H]NMR (DMSO-d_6) δ 8.02 (d, J = 2.1 Hz, H-2′), 7.53 (dd, J = 8.5 and 2.1 Hz, H-6′), 6.92 (d, J = 8.5 Hz, H-5′), 6.45 (d, J = 2.1 Hz, H-8), 6.22 (d, J = 2.1 Hz, H-6), 5.50 (d, J = 7.6 Hz, H-1″), 3.87 (s, OMe); [^{13}C]NMR (DMSO-d_6): δ 156.09 (C-2), 133.14 (C-3), 177.32 (C-4), 161.12 (C-5), 98.61 (C-6), 164.10 (C-7), 93.57 (C-8), 156.28 (C-9), 103.92 (C-10), 121.02 (C-1′), 113.57 (C-2′), 149.35 (C-3′), 146.93 (C-4′), 115.08 (C-5′), 121.84 (C-6′), 55.92 (OMe), 101.41 (C-1″), 74.33 (C-2″), 76.78 (C-3″), 70.27 (C-4″), 77.49 (C-5″), 61.25 (C-6″). These data were in full agreement with those reported by Barbera et al. (1986).

Compound **4** (quercetin-3-O-neohesperidoside) (15 mg) had [^{1}H]NMR δ 7.65 (dd, J = 8.8 and 1.9 Hz, H-6′), 7.52 (d, J = 1.9 Hz, H-2′), 6.82 (d, J = 8.8 Hz, H-5′), 6.18 (d, J = 1.9 Hz, H-6), 5.65 (d, J = 7.3 Hz, H-1″), 5.09 (s, H-1″ ′), 0.86 (d, J = 6.3 Hz, H-6″ ′); [^{13}C]NMR: δ 156.38 (C-2), 133.61 (C-3), 177.37 (C-4), 156.60 (C-5), 98.81 (C-6) 164.03 (C-7), 93.61 (C-8), 161.19 (C-9), 105.23 (C-10), 121.55 (C-1′), 115.37 (C-2′), 144.58 (C-3′), 148.28 (C-4′), 116.50 (C-5′), 121.66 (C-6′), 101.47 (C-1″), 74.18 (C-2″), 76.83 (C-3″), 70.41 (C-4″), 76.08 (C-5″), 67.10 (C-6″), 100.68 (C-1″′), 70.44 (C-2)″′), 70.86 (C-3″′), 72.18 (C-4″′), 68.19 (C-5″′) 17.51 (C-6″′). These data were in full agreement with those reported by Markham et al. (1978).

Compound **5** (isorhamnetin-3-O-neohesperidoside) (12 mg) had [^{1}H]NMR δ 8.01 (d, J = 2.1 Hz, H-2′), 7.54 (dd, J = 8.5 and 2.1 Hz, H-6′), 6.92 (d, J = 8.5 Hz, H-5′), 6.47 (d, J = 2.1 Hz, H-8), 6.22 (d, J = 2.1 Hz, H-6), 5.52 (d, J = 7.6 Hz, H-1″) 5.06 (s, H-1″′), 3.86 (s, OMe), 0.86 (d, J = 6.3

Hz, H-6″′); [^{13}C]NMR: δ 156.08 (C-2), 133.12 (C-3), 177.30 (C-4), 161.15 (C-5), 98.58 (C-6), 164.13 (C-7), 93.56 (C-8), 156.30 (C-9), 103.94 (C-10), 121.00 (C-1′), 113.58 (C-2′), 149.38 (C-3′), 146.95 (C-44′), 115.10 (C-5′), 121.83 (C-6′), 55.90 (OMe), 101.38 (C-1″), 74.39 (C-2″), 76.72 (C-3″), 70.22 (C-4″), 76.11 (C-5″), 77.00 (C-6″), 100.81 (C-1″′), 70.43 (C-2″′), 70.87 (C-3″′), 72.15 (C-4″′), 68.25 (C-5″′), 17.61 (C-6″′). These data agreed with those reported by Markham et al. (1978).

Compund **6** (isorhamnetin-3-O-neohesperidoside-7-O-L-rhamnopiranoside) (mg 24) had [^{1}H]NMR δ 7.87 (d, J = 2.0 Hz, H-2′), 7.52 (dd, J = 8.5 and 2.0 Hz, H-6′), 6.94 (d, J = 8.5 Hz, H-5′), 6.45 (d, J = 2.0 Hz, H-8), 6.43 (d, J = 2.0 Hz, H-6), 5.65 (d, J = 7.1 Hz, H-1″), 5.10 (s, H-1″′), 0.99 (d, J = 6.0 Hz, H-6″′), 0.74 (d, J = 6.3 Hz, H-6⁗); [^{13}C]NMR: δ 156.41 (C-2), 132.51 (C-3), 177.20 (C-4), 161.18 (C-5), 98.70 (C-6), 164.23 (C-7), 93.81 (C-8), 149.38 (C-9), 122.06 (C-1′), 113.30 (C-2′), 146.88 (C-3′), 156.41 (C-4′), 115.23 (C-5′), 121.10 (C-6′), 55.55 (OMe), 100.80 (C-1″), 75.70 (C-2″), 76.88 (C-3″), 70.22 (C-4″), 77.50 (C-5″), 66.73 (C-6″), 100.72 (C-1″′), 70.43 (C-2″′), 70.51 (C-3″′), 71.75 (C-4″′), 68.25 (C-5″′), 17.61 (C-6″′), 98.71 (C-1⁗), 70.43 (C-2⁗), 70.51 (C-3⁗), 71.75 (C-4⁗), 70.21 (C-5⁗), 17.22 (C-6⁗). These data agreed with those reported by Ishida et al. (1988).

RESULTS AND DISCUSSION

The effects of crude extracts and of the most active purified substances from *Typha latifolia*, as well as those of the algicide $CuSO_4$ are shown in Table 1. As can be seen, the ether extract possessed significant biological activity, whereas the chloroform extract was inactive (not shown) and the methanolic extract showed algistatic activity against one strain (T 268 *Coccomyxa elongata*). In order to identify active constituents, bioassay-oriented CC, TLC, and HPLC were performed. Ten active pure compounds were isolated and, on the basis of their physical features, were identified as unsaturated fatty acids, steroids, and flavonol glycosides. β-sitosterol, α-linolenic, and linoleic acid are common compounds in the plant kingdom, occurring in significant amounts in many taxa of lower and higher plants, whereas (20S) 24-methylenlophenol (**1**) has been isolated for the first time by Della Greca et al., (1990), and stigmast-4-ene-3,6-dione (**2**) is a rather uncommon sterol with a cytotoxic activity recently isolated from *Aristolochia versicolor* (Huizhong et al., 1985).

Among the flavonol glycosides **3**, **4**, and **5** are rather common in plants whereas **6** is a new compound with antihemorrhagic activity isolated for the first time by Ishida et al. (1988) in *T. latifolia*. The inhibiting activity of flavonol glycosides was limited to on alga, T 268 *Coccomyxa elongata*, confirming the result obtained with the crude methanol extract. Moreover, amounts of

TABLE 1. INHIBITION OF ALGAL GROWTH BY *T. latifolia*[a]

Phylum	Strain	Species	Crude extracts (0.5 mg)		Compounds isolated from Et_2O extract						$CuSO_4$	
			Et_2O	MeOH	A[b]	B	C	D[b]	E[b]	F	1 μmol	0.5 μmol
Cyanochloronta	T 584	*Nostoc commune*	−	−	−	−	−	−	−	−	++	+
	T 625	*Synecococcus leopoliensis*	++	−	−	+	+++	++	+	+	++	++
	T 1444	*Anabaena flos-aquae*	++	−	−	+	++	++	+	+	++	++
	T 1580	*Phormidium autumnale*	++	−	++	−	−	−	−	−	++	+
	T 1816	*Porphyrosiphon notarisii*	++	−	−	−	−	++	+	++	++	+
	T 1824	*Aulosira terrestre*	−	−	−	−	−	−	−	−	++	++
	T 2349	*Scytonema hofmanni*	++	−	−	−	−	++	−	−	++	+
Rhodophycophyta	T 755	*Porphyridium aerugineum*	+	−	−	+	−	+	−	+	+	−
Chrysophycophyta	T 671	*Navicula pelliculosa*	+	−	+	−	−	+	−	−	++	+
Chlorophycophyta	C 211-8h	*Chlorella emersonii*	+	−	+	−	+	−	−	−	+++	+++
	C 249-1	*Muriella aurantiaca*	+	−	−	−	+	+	+	−	+	−
	C 379-1c	*Stichococcus bacillaris*	+	−	+	−	−	+	−	−	+	−
	C 1224-5a	*Euglena gracilis*	−	−	−	−	−	−	−	−	−	−
	T 268	*Coccomyxa elongata*	+	±	−	+	−	−	−	−	+++	−
	T 293	*Chlamydomonas sphagnophila*	−	−	−	−	−	−	−	−	+	−
	T 397	*Chlorella vulgaris*	+	−	+	−	+	−	−	−	++	−
	T 1075	*Closterium acerosum*	+	−	−	−	−	+	−	−	+	−
	T1648	*Selenastrum capricornutum*	+	−	−	−	−	+	−	−	++	−

[a]A, β-sitosterol 90.7 μmol); B, (20S) 24-methylenlophenol (0.7 μmol); C, stigmast-4-ene-3, 6-dione (0.7 μmol); D, α-linolenic acid (1.0 μmol); E, linoleic acid (1.0 μmol); F, unidentified $C_{18:2}$ (1.0 μmol); +, diameter of inhibition 7–14 mm; ++, diameter of inhibition 15–23 mm; +++, diameter of inhibition 23 mm; −, no inhibition; ±, algistatic effect.

[b]Commercially available product showed identical activity.

0.5 mol of these compounds caused only a transient inhibition zone, which, after five days, disappeared almost completely. Probably the flavonol glycosides cause only a reduction of the growth rate of the algae during the first days after their addition. In this respect, at the amounts used, they can be considered algistatic and not algicidal.

In contrast, the inhibitory activity of fatty acids and sterols was similar to that shown by $CuSO_4$ (0.5 μmol). The most active compounds were stigmast-4-ene-3,6-dione and α-linolenic aacid, which produced inhibition zones on five and ten algal strains, respectively. Noteworthy is the sensitivity of blue-green algae (particularly T 625 *Sinechococcus leopolensis*, and T 1444 *Anabaena flos-aquae*) to the compounds tested. The selective inhibitory action of aquatic plants on blue-green algae was reported for the first time by Hasler and Jones (1949), who found that *Potamogeton natans* and *Anacharis canadensis* inhibited the growth of blue-green algae. Subsequently, Kogan and Chinnova (1972) pointed out that growth of blue-green algae was inhibited by *Ceratophyllum demersum*, while the growth of some green algae was not affected by the same plant (Kogan et al., 1972). Finally, Van Aller et al. (1985) showed the inhibiting effects of compounds isolated from *Eleocharis microcarpa* on a number of genera of blue-green algae, among which were listed *Anabaena* and *Anacystis* (synonymous with *Synechococcus*).

Few data on the inhibitory action of steroids are available (Rice, 1984), and only one report indicates that steroids can have a strong antimicrobial activity (Evenari, 1949). On the other hand, there is increasing evidence of the allelopathic properties of long-chain fatty acids on algae reported by Proctor (1957) and confirmed by Kroes (1972). McCraken et al. (1980) showed that the material used by Proctor consisted of a mixture of at least 15 free fatty acids. Among the three active fatty acids from *T. latifolia*, the most effective against algae was α-linolenic acid, which supports the findings of Kakisawa et al. (1988), who showed that toxicity of fatty acids increases with an increase in double bonds.

Recently, we have found antialgal activity of some $C_{18:3}$ hydroxy fatty acids in *Pistia stratiotes* (not shown). This kind of compound is produced through hydroperoxide intermediates either by lipoxygenase oxidation (Funk et al., 1976) or by autooxidation (Chan and Levett, 1977) of α-linolenic acid. In order to verify if, in our experimental conditions, the antialgal activity is due to α-linolenic acid and/or to its oxidation products, samples of agarized medium from a Petri dish containing α-linolenic acid were analyzed after three days' exposure to air and artificial light at 25°C; only 10% of the α-linolenic acid was recovered together with a mixture of nine fatty acid hydroperoxides, which also showed algal inhibition. Moreover, when a nontoxic antioxidant agent such as l-ascorbic acid was added to the medium, a reduced algal growth inhibition was observed, and in this condition 60% of the α-linolenic acid was recovered.

These preliminary results seem to indicate that the antialgal activity is due more to the oxidation products than to α-linolenic acid.

Investigations in natural habitats in which *T. latifolia* occurs are required to confirm the in vitro inhibiting action observed against some blue-green microalgae. In this respect, it is interesting to note that Fischer and Quijano (1985), in analyzing the water washes of undamaged leaves of some plants, have found sterols and fatty acids in concentrations comparable with those used in a typical laboratory bioassay. These data suggest the possibility that the allelopathic effects in aquatic ecosystems could be determined also by water-insoluble compounds, such as steroids and fatty acids.

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OLFACTORY SENSITIVITY OF TWO SYMPATRIC SPECIES OF RICE LEAF FOLDERS (LEPIDOPTERA: PYRALIDAE) TO PLANT VOLATILES

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Abstract—Electroantennograms (EAGs) were recorded from male and female moths of two sympatric leaf folder species, *Cnaphalocrocis medinalis* (Guenée) and *Marasmia patnalis* Bradley (Lepidoptera: Pyralidae) to 91 volatile chemicals of plant origin. Responses of both leaf folder species were similar to all compounds except to three monoterpenes—β-myrcene, menthone, and isomenthone—and two sesquiterpenes—*cis*-nerolidol and isophytol. Response of *M. patnalis*, an oligophagous leaf folder, to these compounds was higher compared with that of polyphagous *C. medinalis*. EAG responses of males to saturated and unsaturated aliphatic aldehydes were significantly higher than those of conspecific females in both species. A higher response of *C. medinalis* males also was observed for 1-nonanol, 3-nonen-2-one, and *trans,trans*-2,6-dimethyl-2,4,6-octatriene. In contrast, females of both species responded more to monoterpenes, borneol, isoborneol, and fenchyl alchohol. Response of *C. medinalis* female was higher for terpinen-4-ol, carveol, dihydrocarveol, (−)-myrtenal, and perillaldehyde. In both species and sexes, high EAG responses were recorded for compounds of the green leaf odor complex. EAG responses to nonanal and hexanal were maximum among the aliphatic aldehydes while 1-hexanol elicited the highest response among the alcohols tested. EAG responses to terpene compounds—citronellal, α-terpineol, and (−)-myrtenal—were equal to the response to 1-hexanol. While all compounds tested elicited a negative potential, thymol and carvacrol elicited a positive EAG potential. The EAG data are discussed with regard

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to the role of host plant volatiles in host selection by the two leaf folder species.

Key Words—*Cnaphalocrocis medinalis*, electroantennogram, green leaf volatiles, host-plant resistance, Lepidoptera, *Marasmia patnalis*, olfaction, plant volatiles, Pyralidae, rice leaf folder.

INTRODUCTION

Rice leaf folders (Lepidoptera: Pyralidae), which were generally considered pests of minor importance, have increased in abundance since the introduction of high-yielding rice varieties (Waldbauer and Marciano, 1979), warranting the development of resistant rice varieties and other effective control measures. *Cnaphalocrocis medinalis* (Guenée) and *Marasmia patnalis* Bradley are two sympatric leaf folder species that occur widely in ricefields of South and Southeast Asia and were, until recently, not distinguished as different species because of their morphological similarity (Bradley, 1981; Joshi et al., 1986). The host range of *C. medinalis* includes several graminaceous plants and weeds (Joshi et al., 1987), but the most suitable host plant is reported to be rice (Yadava et al., 1972). Although not much information is available on the host range of *M. patnalis*, recent studies have indicated that this species has a much narrower host range (Z.R. Khan and M.L. Abenes, unpublished data) than *C. medinalis*. Host selection in rice leaf folder is primarily achieved by adults that are active fliers. A number of insects have been reported to be sensitive to general green plant volatiles and volatiles that are typical of their host plants (Visser, 1979; Guerin and Visser, 1980; Yan and Visser, 1982; Dickens, 1984; Dickens and Boldt, 1985; Whitehead, 1986; Light and Jang, 1987; Hansson et al., 1989). Sensitivity to plant volatiles has been shown to differ between generalist and specialist grasshopper (Blust and Hopkins, 1987) and also between populations of the same species associated with different host plants (Honda et al., 1986). Understanding the olfactory sensitivity of insects to plant volatiles may help in the identification of semiochemicals responsible for attraction or repulsion of an insect (Metcalf, 1986) and may provide a basis for breeding resistant varieties (Liu et al., 1988).

In the present study, we examined the olfactory sensitivity of males and females of *C. medinalis* and *M. patnalis* to a spectrum of volatile chemicals by using the electroantennogram (EAG) technique in order to identify volatile chemicals that may affect the behavior of these insects. We also have discussed the sexual and species differences in the response profile in relation to the host-plant relations of these two species.

METHODS AND MATERIALS

Insects and Plants. *C. medinalis* and *M. patnalis* were reared on susceptible IR36 rice plants in a greenhouse following the rearing method developed at IRRI (Walbauer and Marciano, 1979). Pupae were collected and maintained in an incubator at 27 ± 2°C, and 12:12 light–dark photoperiod, with the scotophase starting at 0800 hr. Adults were collected immediately after their emergence. The emerged moths were given 25% honey solution. Experiments were conducted on 4-day-old females and 2-day-old males.

Antennal Preparation. A moth temporarily anesthetized with CO_2 was immobilized in a polypropylene pipet with paraffin wax. The insect's head and a part of the prothorax protruded out of the narrow end of the pipet. The right antenna of the moth was snipped off at the base to permit easy access of the indifferent electrode to the base of the recording antenna. A wad of cotton dipped in distilled water was inserted onto the wider end of the pipet to prevent rapid desiccation of the moth. Moths were allowed to recover for 30 min before conducting the experiments.

The antenna was inserted into the tip of a glass pipet (20 μm diam) with a chlorinated silver wire (Ag–AgCl) electrode (different electrode) filled with saline solution (NaCl, 3.75 g; $CaCl_2$, 0.105 g; KCl, 0.175 g; $NaHCO_3$, 0.100 g; H_2O, 500 ml) (Roelofs, 1976) containing 10% by volume of polyvinylpyrrolidone K90 (Fluka AG, Buchs, Switzerland). The Ag–AgCl wires were connected to the input of an amplifier (type AM-05, Syntech, Hilversum, The Netherlands), the output of which was displayed on an oscilloscope screen (HM-205). The EAG peak heights (−mV) were measured directly from the oscilloscope.

An experimental antenna was continuously bathed in a stream of activated-charcoal-filtered air (40 ml/sec) delivered by a 12-cm stainless-steel tube positioned 2 cm from the antennal preparation. The stimulus was applied from a Pasteur pipet. The tip of the pipet was introduced into a small hole located on the side of the steel tube. The other end of the pipet was placed in an injection device (Syntech), which delivered 1 ml of air. The duration of the stimulus pulse was 1 sec.

A funnel (10 cm diam) connected to a vacuum line was positioned 10 cm behind the antennal preparation to evacuate the area of any volatile compounds and was operated for 2 min in between stimulations. The antenna was stimulated at 2-min intervals with five test chemicals, each followed and preceded with a standard (100 μg of 1-hexanol) and control (10 μl of heptane) (Light and Jang, 1987). The antennal preparation remained in good condition for several hours, and the responsiveness of the antenna decreased gradually.

Chemicals. Volatile chemicals of plant origin tested and their source and

purity are presented in Table 1. The chemicals were dissolved in spectrometric grade heptane to produce 10 mg/ml solutions, and the vials numbered serially. During the experiment, these chemicals were referred to by those numbers.

Experimental Protocol. From the test solutions, cartridges were produced for each compound by pipetting 10-μl aliquots onto 6-cm $\times$ 0.5-cm filter paper (previously washed with HPLC grade hexane and oven-dried at 60°C). The solvent was allowed to evaporate for 1 min, and the filter paper was inserted into individual Pasteur pipets. Cartridges were always prepared prior to stimulation in a room different from the one housing the EAG preparation and were transferred with the tips sealed with parafilm. EAGs were recorded randomly on different days from male and female moths of the two species. Likewise, chemicals were randomly tested. All chemicals were tested on a species and/or sex on a given day. EAGs from four males and four females of each species were recorded.

EAGs to test compounds were evaluated by measuring the maximum amplitude of negative deflection (−mV) elicited by a given stimulus, and then subtracting the amplitude of the response to the accompanying solvent control. The millivolt responses to all compounds were coverted to percentage values of the response to the accompanying standard (100 μg of 1-hexanol), as used

TABLE 1. SOURCE AND PURITY OF CHEMICALS USED IN ELECTROANTENNOGRAM STUDIES

Chemicals	Chemical purity (%)	Source[a]	Compound	Chemical purity (%)	Source
Green leaf volatile chemicals			Green leaf volatile chemicals		
Alipathic alcohols			Alipathic aldehydes		
1-Pentanol	95	A	Hexanal	>90	B
1-Hexanol	>90	B	Heptanal	95	A
1-Nonanol	>90	B	Nonanal	95	A
1-Tetradecanol	97	A	*trans*, *trans*-2,4-hexadienal	95	A
1-Heptanol[b]	98	A			
3-Pentanol	98	A	*trans*-2-Hexenal	99	A
cis-2-Penten-1-ol	95	A	trans, *trans*-2,4-decadienal	Tech	A
cis-2-Hexen-1-ol[b]	92	A			
trans-2-Hexen-1-ol	97	A	*trans*, *trans*-2,4-nonadienal	90	A
cis-3-Hexen-1-ol	98	B			
trans-3-Hexen-1-ol	98	A	*trans*-2-Octenal	94	A
trans-2-Heptanol	96	A			
1-Hexen-3-ol	98	A			
Alipathic hydrocarbons			Alipathic ketones		
trans, *trans*-2,6-dimethyl-2,4,6-octatriene[b]	Tech	A	2-Pentanone	97	A
			3-Penten-2-one	Tech	A
			2-Heptanone[b]	98	A
Octane[b]	99	A	3-Nonen-2-one	95	A

TABLE 1. Continued.

Chemicals	Chemical purity (%)	Source[a]
Monoterpenes		
Alcohol		
(−)-Borneol	>99	C
Isoborneol	>99	C
(−)-Carveol	97	A
Dihydrocarveol	>99	C
Fenchyl alcohol	>99	C
D-Isomenthol	>99	C
Isopulegol	>99	C
trans-p-menth-6-ene-2,8 diol	95	A
Terpinene-4-ol[b]	97	A
α-Terpineol[b]	98	A
p-Menth-1-en-9-ol	97	A
trans-Myrtanol	>99	C
Geraniol	>99	C
Linalool[b]	97	A
L-Citronellol	>99	C
Thujyl alcohol	>99	C
Menthol	99	A
Carvacrol	98	A
Aldehydes		
(−)-Myrtenal	>99	C
Perillaldehyde	>99	C
Citronellal	>99	C
Hydrocarbons		
2-Carene	95	A
3-Carene[b]	95	A
α-Terpinene[b]	85	A
γ-Terpinene[b]	95	A
Terpinolene[b]	>99	C
Limonene[b]	>90	C
Camphene	>99	C
α-Pinene[b]	99	A
β-Myrcene[b]	>90	B
Sabinene[b]		

Compound	Chemical purity (%)	Source
Monoterpenes		
Ketones		
Carvenone[b]	>99	C
Dihydrocarvone	>99	C
Isomenthone	>99	C
Menthone	95	A
Piperitone	>99	C
Thujone	>99	C
α + β-Thujone[c]	>99	C
β-thujaplicin	>99	C
Ester		
Terpenyl acetate	>99	C
Epoxide		
Cineole	>99	B
Aromatic Compounds		
Cumene	99	A
p-Cymene[b]	96	A
Estragol	>99	C
Safrol	97	A
Ethyl benzoate	99	A
Methyl benzoate[b]	99	A
Ethyl salicylate	99	A
Methyl salicylate[b]	99	A
Eugenol	90	A
Thymol	>99	C
Acetyl eugenol	>99	C
Benzyl alcohol	>99	A
Sesquiterpenes		
(−)-*trans*-caryophyllene[b]	>90	B
Cedrol	>99	C
Farnesol	>99	C
Isophytol	>99	C
cis-nerolidol	>99	C
(*cis* + *trans*)-Nerolidol[c]	>99	C
Cedrenol	>99	C
Phytol	80–90	C
α-(−)-Bisabolol	>99	C
Irone	>99	C
cis-Jasmone	>99	C

[a] A = Aldrich Chemical Company, Milwaukee, Wisconsin; B = Sigma Chemicals Company, St. Louis, Missouri; C = Target Synthesis Extraction, BP100 33701 Therigrace, France.
[b] Presence in rice plant confirmed (P. Caballero et al., unpublished data).
[c] 1 : 1 ratio.

in other EAG studies on insect olfaction (Guerin and Visser, 1980; Dickens, 1984; Dickens and Boldt, 1985; Light and Jang, 1987). Data on the EAG response to a particular class of chemicals (saturated aldehydes, unsaturated aldehydes, etc.) were analyzed by three-factor ANOVA to distinguish the effects of sex, species, and compounds. Multiple comparisons were done by Tukey's Honestly Significant Difference Test (Zar, 1984) at $P < 0.05$. Within a class of chemicals, if either sex or species factor is significant, the means for both sexes of a species (when species factor is significant) or the same sex (when sex factor is significant) are given in the parenthesis in the results section. Means of compounds in different chemical classes are compared on the basis of magnitude only.

RESULTS

Responses to Standard

The absolute EAG responses to the standard, 1-hexanol, elicited a significantly higher EAG amplitude in *C. medinalis* males (3.75 ± 0.5) than the conspecific females (2.7 ± 0.4) and *M. patnalis* females (2.55 ± 0.10) and males (2.0 ± 0.2) ($P < 0.05$). The values for the first stimulation with the standard was considered in this analysis.

Response to Green Leaf Volatile Chemicals

Saturated Aldehydes. The response profiles to saturated aldehydes of both the species were similar. Male responses were significantly higher than those of the females (sex factor significant, $F_{1,33} = 6.03$, $P < 0.019$). Response to nonanal and hexanal (mean of both sexes and species = 176% and 160%, respectively) were significantly higher than response to heptanal (145%) (Figure 1A).

Unsaturated Aldehydes. No species difference was observed in the responses of unsaturated aldehydes. When the response profiles of the two sexes were compared, the relative response of the males (113%) was higher than that of the females (101%) (sex factor significant, $F_{1,57} = 6.27$, $P < 0.015$)(Figure 1B). Response to chemicals differed significantly with *trans*-2-octenal (140%) eliciting the highest response followed by *trans*,*trans*-2,4-nonadienal (112%). Lowest response was recorded for 2,4-hexadienal (87%) and *trans*-2-hexenal (96%) (Figure 1B).

Saturated Alcohols. Significant differences in the response profiles of males and females were observed for 1-nonanol in *C. medinalis* and for 1-tetradecanol and 1-pentadecanol in *M. patnalis* (Figure 2A). In both cases, the male responses were higher than those of females. Among the saturated alchohols, 1-hexanol

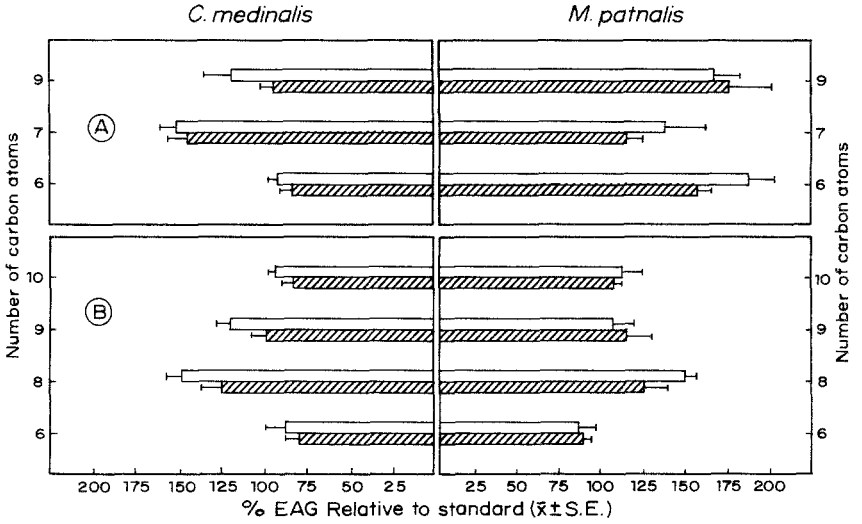


FIG. 1. Mean EAG responses of females (empty bars) and males (lined bars) of *C. medinalis* and *M. patnalis* to (A) saturated aldehydes and (B) unsaturated aldehydes of different carbon chain lengths.

and 1-heptanol elicited the highest response in both species and sexes. Responses to all other compounds in this group were lower (Figure 2A).

Unsaturated Alcohols. Responses of both species and sexes were similar in this group of chemicals. However, significant differences were observed among the effects of different chemicals. *trans*-2-Hepten-1-ol elicited the highest response followed by *cis*-2-hexen-1-ol, *trans*-2-hexen-1-ol, *cis*-3-hexen-1-ol, *trans*-3-hexen-1-ol, and *cis*-2-penten-1-ol (Figure 2B).

6-Carbon Alcohols and Aldehydes. Hexanal, the saturated aldehyde, produced the highest EAG response, while 1-hexen-3-ol, a secondary alcohol, produced only 10–30% response to the standard. Unsaturation of aldehydes reduced the EAG response, but it did not significantly reduce the response to alcohols (Figure 3).

Ketones. Among the four compounds in this group, a significantly high response was observed for 3-nonen-2-one in both species and sexes. While no sexual difference in the response was observed for *M. patnalis*, the response of *C. medinalis* male moths (Figure 4) to this compound was significantly higher than that of females.

Hydrocarbons. Response of *C. medinalis* males to stimulation with *trans,trans*-2,6-dimethyl-2,4,6-ocatriene was significantly higher than that of females (Figure 4). No difference in the responses between the two sexes was observed in *M. patnalis* (Figure 4). Octane did not elicit a significant EAG response in both species.

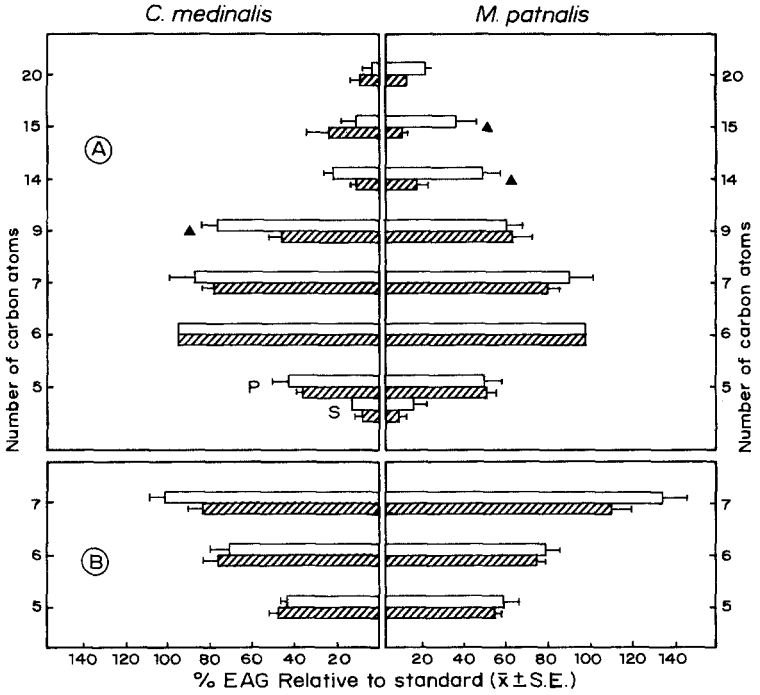


FIG. 2. Mean EAG responses of females (empty bars) and males (lined bars) of *C. medinalis* and *M. patnalis* to (A) saturated primary and (B) unsaturated alcohols of different carbon chain lengths. (▲) indicates significant difference in responses of conspecific males and females.

Monoterpenes

Alcohols. There was a significant interaction between sex, species, and compound in the response to this group of chemicals. In both species, female responses to borneol, isoborneol, fenchyl alcohol, thujyl alcohol, and *trans*-(−)-myrtanol were significantly higher than those of the males (Figure 5) ($P < 0.05$). Response of *C. medinalis* females was higher than that of the males for terpinen-4-ol, while male *M. patnalis* produced a higher relative EAG response than the female for the same compound. Response of *C. medinalis* males was lower than its females for carveol and dihydrocarveol, while such sexual differences were not observable in *M. patnalis* (Figure 5). α-Terpineol and linalool were two compounds of this group that elicited a response as high as that of the standard (Figure 5) in the males and females of the two leaf folder species.

Hydrocarbons. There was a significant interaction between species and

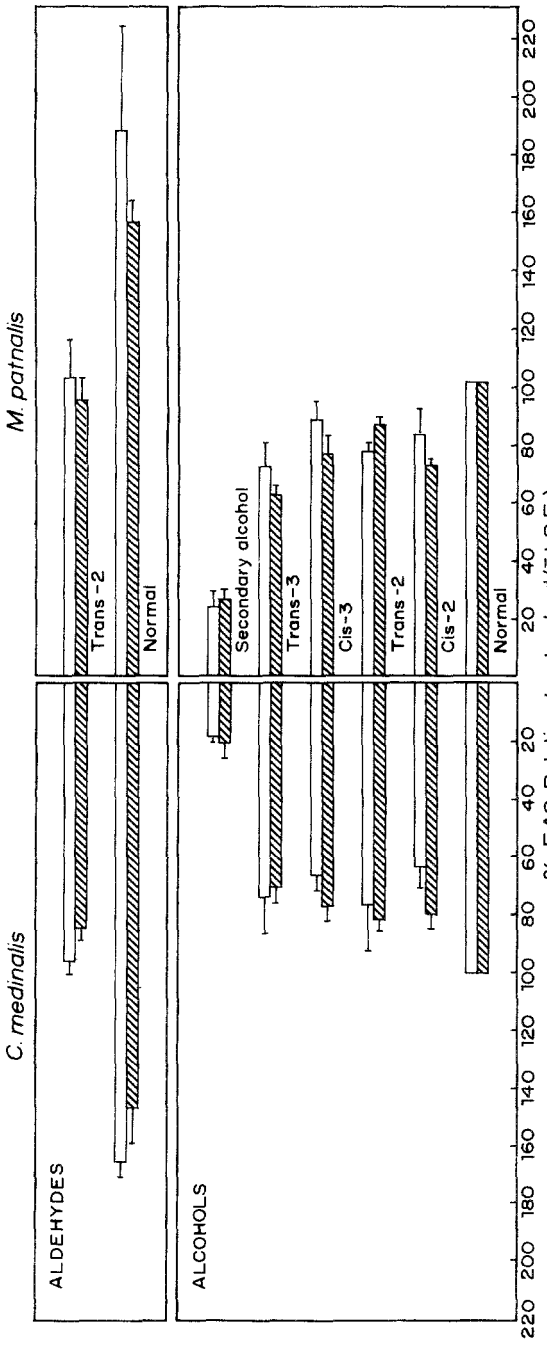


FIG. 3. Mean EAG responses of females (empty bars) and males (lined bars) of *C. medinalis* and *M. patnalis* to six-carbon alcohols and aldehydes.

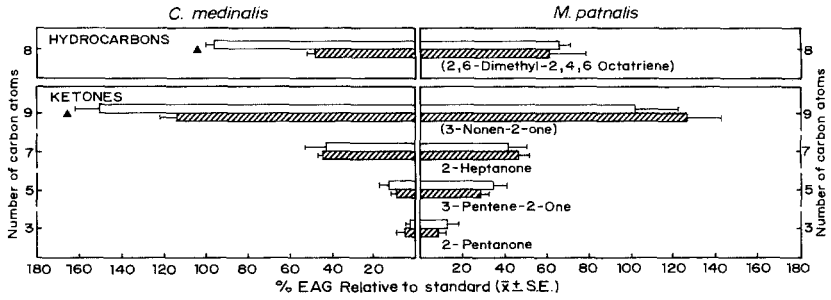


FIG. 4. Mean EAG responses of females (empty bars) and males (lined bars) of *C. medinalis* and *M. patnalis* to aliphatic ketones and hydrocarbons.

compounds in the responses to terpene hydrocarbons. Comparison of the means indicated that the difference in the response was only to β-myrcene, with *M. patnalis* (55.5%) responding more than *C. medinalis* (32.5%). β-Myrcene also elicited the highest response in both species (Figure 6).

Ketones, Esters, Epoxides, and Aldehydes. Response of *M. patnalis* was higher than that of *C. medinalis* to menthone (80.5% and 56%, respectively) and isomenthone (73% and 42%, respectively). Dihydrocarvone elicited higher EAG response in both species than did (*R*)-(−)-carvone. Responses to ester, terpenyl acetate, and epoxide (cineole) were low and of the order of only about 10–20% (Figure 7). The aldehydes (−)-myrtenal and perillaldehyde elicited higher EAG responses in females than in males of *C. medinalis*, while no sexual difference in the responses to these compounds were observed for *M. patnalis*. The response of female *C. medinalis* to (−)-myrtenal was as high as that of the standard (Figure 7), while citronellal elicited a response comparable to that of the standard in both sexes of the two species.

Sesquiterpenes

Among the 11 sesquiterpenes, the response of *M. patnalis* was significantly higher than that of *C. medinalis* for *cis*-nerolidol (54% and 28.5%) and isophytol (27% and 7%, respectively) (Figure 8). Response of *M. patnalis* to (*cis* + *trans*)-nerolidol was also higher than that of *C. medinalis*, but the difference was not statistically significant. In general, the response to the other sesquiterpenes remained low (<20%) in both species (Figure 8) except for *cis*-jasmone.

Aromatic Compounds

Estragol elicited a significantly higher response in the female *C. medinalis* than in males (Figure 9). Ethyl benzoate, methyl benzoate, ethyl salicylate,

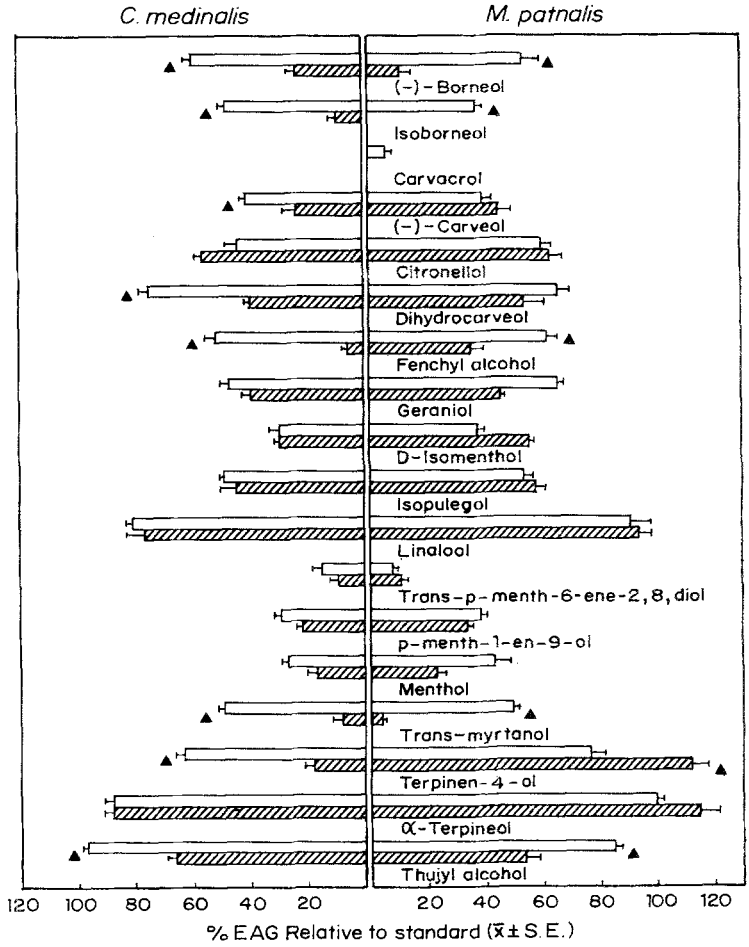


FIG. 5. Mean EAG responses of females (empty bars) and males (lined bars) of *C. medinalis* and *M. patnalis* to monoterpenes (alcohols). (▲) indicates significant difference in response of conspecific males and females.

methyl salicylate, and safrol elicited a response ranging from 40% to 50% of the standard in both species of leaf folders (Figure 9).

Shape of EAG Signals

In general, the shape of the EAG signals in response to stimulation with plant volatiles was characterized by a fast negative deflection after the onset of stimulation, followed by a slow return to the baseline (Figure 10A). However,

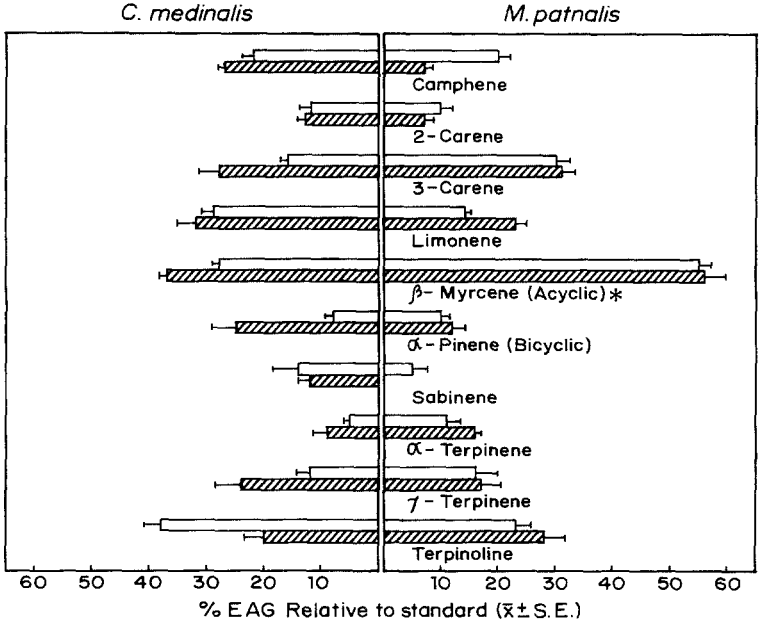


FIG. 6. Mean EAG responses of females (empty bars) and males (lined bars) of *C. medinalis* and *M. patnalis* to monoterpenes (hydrocarbons). (*) indicates significant difference in the responses of the two species.

stimulation with carvacrol and thymol elicited a small negative deflection but a very large positive deflection (Figures 10B and C).

DISCUSSION

Insects possess intimate and subtle relationships with their host plants. Recently, several studies have demonstrated the importance of plant chemical factors in determining the susceptibility or resistance of rice varieties to insect pests (Khan and Saxena 1985; Khan et al., 1988; Saxena and Okech, 1985; Saxena, 1986). Identification of these semiochemicals will help in strengthening the approaches for breeding resistant rice varieties. The present study elucidates the importance of plant volatiles involved in leaf folder–rice plant interactions.

The EAG responses of both *C. medinalis* and *M. patnalis* were similar to most of the volatile chemicals except to three monoterpenes (β-myrcene, menthone, and isomenthone) and two sesquiterpenes (*cis*-nerolidol and isophytol). The response of *M. patnalis* to these chemicals was higher compared with that of *C. medinalis*. Studies on the comparison of EAG responses of different spe-

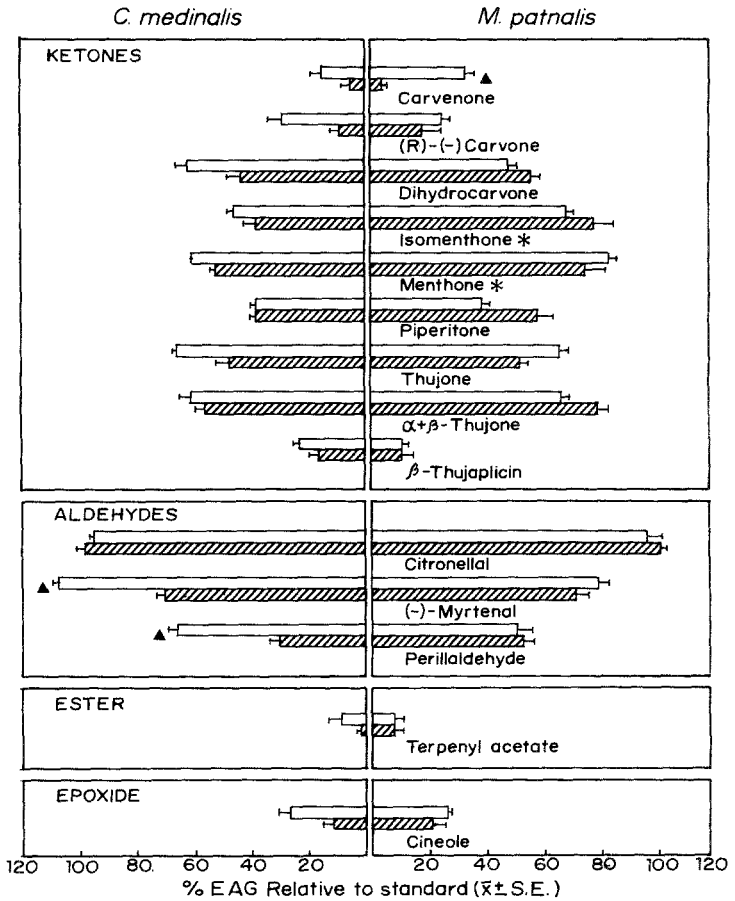


FIG. 7. Mean EAG responses of females (empty bars) and males (lined bars) of *C. medinalis* and *M. patnalis* to monoterpenes (ketones, aldehydes, ester, and epoxide). (*) indicates significant differences in the responses of the two species; (▲) indicates significant difference in responses of conspecific males and females.

cies or genera have indicated similarity in the responses of the test species to plant volatiles (Van der Pers, 1981) or differences in the responses to specific chemicals (Blust and Hopkins, 1987) reflecting the host-plant association of the test species. Even within the same species, differences in the EAG profiles to plant volatiles of populations associated with different host plants have been reported (Honda et al., 1986). Since both *C. medinalis* and *M. patnalis* utilize rice plants and other graminaceous plants as their hosts, the remarkable similarity in their EAG profiles is not very surprising. As *C. medinalis* has a wider host range within the graminae than *M. patnalis*, the higher responses of *M.*

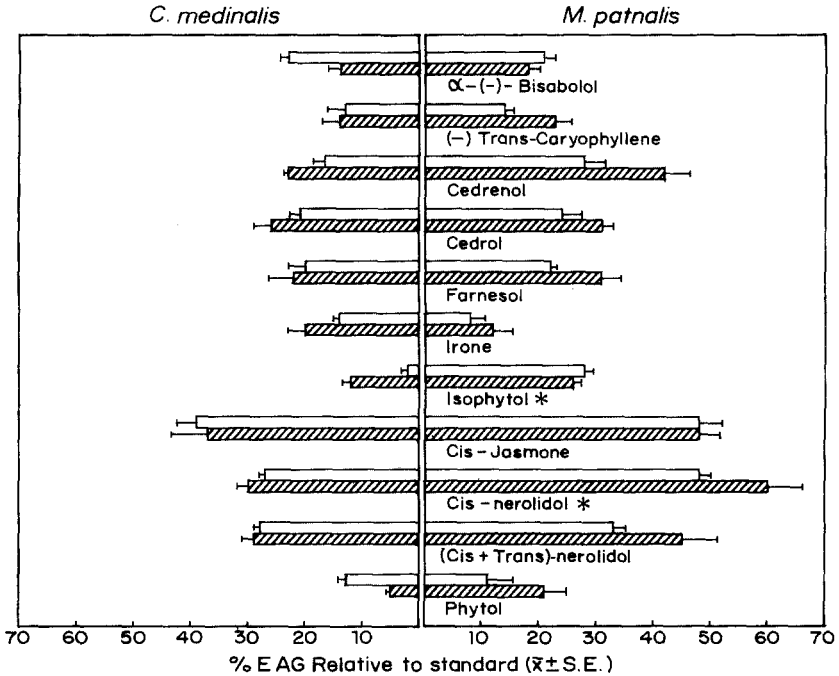


FIG. 8. Mean EAG responses of females (empty bars) and males (lined bars) of *C. medinalis* and *M. patnalis* to sesquiterpenes. (*) indicates significant difference in the responses of the two species.

patnalis to some of the volatile chemicals also reflect a relatively narrower host range of this species.

In general, the relative responses of the males of both leaf folder species were higher for primary straight-chain saturated and unsaturated aldehydes, while female responses were higher for terpene compounds. Males of *C. medinalis* also showed higher response to three green odor compounds—1-nonanol, 3-nonen-2-one, and *trans,trans*-2,6-dimethyl-2,4,6-ocatriene—but elicited lower response than did conspecific females to some monoterpene alchohols and aldehydes (Figure 5). Higher response of males to plant volatile chemicals has been reported for several insect species (Guering and Visser, 1980; Dickens, 1984; Hansson et al., 1989; Blust and Hopkins, 1987; Honda et al., 1986). On the other hand, higher female responses also were recorded for three of the 35 green odor compounds tested on *Dacus dorsalis* Hendel (Light and Jang, 1987). Similarity in the sensitivity of males and females to several plant volatiles suggests a common ecological need for olfactory orientation and assessment of host plants and/or habitat by both sexes (Light and Jang, 1987). The higher responsiveness of males to compounds in the green leaf odor complex

suggests that this group of chemicals may serve as long-range attractants for males locating the female's host habitat. Upon arrival in the habitat, males and females may utilize the perceived resources in either a similar (e.g., foraging for food) or dissimilar manner (e.g., locating females ready to mate in the case of males or locating suitable oviposition sites in the case of females). The higher response to some of the terpene compounds of the females as compared with that of the males suggests that these compounds may be involved in closer-range behaviors related to oviposition.

The highest response to primary alcohols of different carbon chain lengths was observed for 6-carbon alcohol (1-hexanol) in both leaf folder species and was similar to the responses of several other insect species (Visser, 1979; Kozlowski and Visser, 1981; Dickens, 1984; Yan and Visser, 1982; Light and Jang, 1987). Among the primary aldehydes tested, peak responses were observed in both species for C_6 and C_9 aldehydes; response to C_7 aldehyde was significantly lower, suggesting a bimodal peak in the response profile to saturated aldehydes. A similar bimodal response to aldehydes of different carbon chain lengths also has been reported in the males of another pyralid moth *Conogethes punctiferalis* Guenée (Honda et al., 1986). However, the majority of

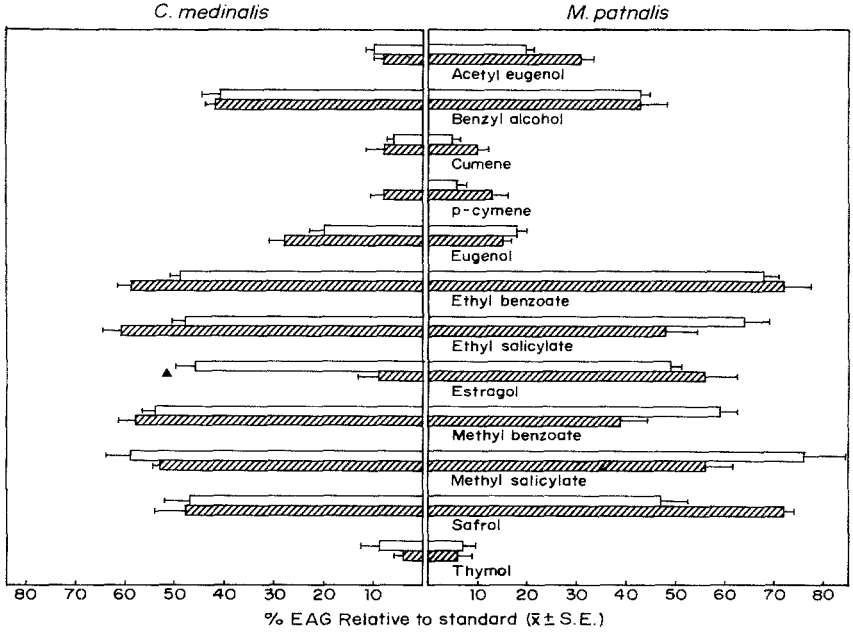


FIG. 9. Mean EAG responses of females (empty bars) and males (lined bars) of *C. medinalis* and *M. patnalis* to aromatic compounds. (▲) indicates significant difference in responses of conspecific males and females.

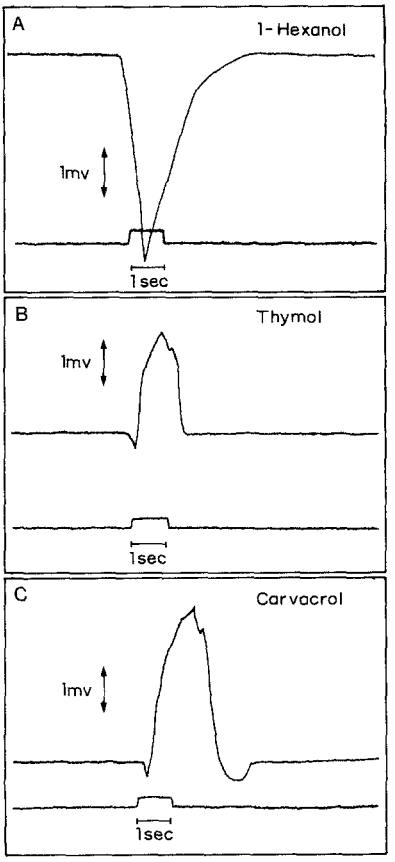


FIG. 10. Shape of EAG signals elicited by plant volatiles. (A) Typical EAG signal elicited by 1-hexanol characterized by a fast negative potential and slow return to baseline. (B and C) EAG signals elicited by thymol and carvacrol, characterized by small negative potential and large positive potential.

the EAG–plant volatile studies have reported responsiveness to aldehyde peaks for C_7 with this ranking: $C_7 > C_8 > C_9 > C_6$ (Guerin and Stadler, 1982; Guerin et al., 1983a,b; Van der Pers, 1981; Dickens, 1984; Dickens and Boldt, 1985).

EAG responses to certain monounsaturated alcohols, aldehydes, and acetates have been reported to exceed those of their saturated analogs (Visser, 1983, 1986). In contrast, the leaf folder adults respond more to saturated aldehydes than to corresponding unsaturated analogs while unsaturation of alcohols did not alter the response.

Aliphatic aldehydes have been reported to be either equal to or greater than alcohols of corresponding carbon-chain length in their stimulatory potency to several insects (Guerin and Visser, 1980; Guerin and Stadler, 1982; Guerin et al., 1983a,b); in other insects, either aldehydes (Dickens, 1984; Dickens and Boldt, 1985) or alcohols (Guerin and Stadler, 1982; Visser, 1979; Kozlowski and Visser, 1981; Van der Pers, 1981) have been reported to be selectively stimulatory. In leaf folders, response to C_6 and C_9 saturated aldehydes exceeded response to corresponding alcohols in both species. Thus, our results are similar to those obtained with the coleopterans *Anthonomus grandis* Boheman (Dickens, 1984) and *Trirhabda bacharides* (Weber) (Dickens and Boldt, 1986). Response to the g-carbon ketone (3-nonen-2-one) was higher than the response to corresponding alcohol (1-nonanol), but it was less than the response to aldehyde; the hydrocarbon (*trans,trans*-2,6-dimethyl-2,4,6-ocatriene) was found to be selectively stimulatory to *C. medinalis* males only.

Monoterpenes with functional groups of alcohols, aldehydes, and ketones, were more stimulatory than hydrocarbons, esters, and epoxides. Oxygenated monoterpenes have been reported to elicit greater EAGs than hydrocarbons in several other insect species (Visser, 1979; Kozlowski and Visser, 1981; Guerin and Visser, 1980; Dickens, 1984; Dickens and Boldt, 1985). Responses of both the species to linalool and α-terpineol (monoterpenes with alcohol moiety) and citronellal (with an aldehyde moiety) were as high as that of the standard, 1-hexanol, while (−)-myrtenal (with an aldehyde moiety) elicited a response equal to the standard only in the females of *C. medinalis*. Linalool and α-terpineol have been identified in the headspace collection of rice plants (P. Caballero et al., unpublished data), but the presence in rice of the other terpene compounds that elicited high EAG response is not known. Among the aromatic compounds, ethyl benzoate, methyl benzoate, ethyl salicylate, methyl salicylate, estragol, and safrol elicited high EAG responses in both species. Obata et al. (1983) identified ethyl benzoate, methyl benzoate, ethyl salicylate, and methyl salicylate in the ether extracts of a susceptible rice plant.

The sesquiterpene that elicited the highest response from both species was *cis*-nerolidol, a mixture of its optical isomer, and *cis*-jasmone. However, the level of response was only 30–50% of that of 1-hexanol. *trans*-Caryophyllene, one of the components of the headspace collection of rice plants (P. Caballero, unpublished data) elicited very weak EAG signals.

Contreras et al. (1989) reported that it was possible to predict if a chemical was an attractant or repellent on the basis of the EAG pattern displayed by the chemical. The authors found that, in *Periplaneta americana*, chemicals showing negative EAG were attractants at lower concentrations while those showing positive EAG were repellents. Thymol and carvacrol, which elicited a positive EAG in both sexes of the two leaf folder species in the present study, also were

found to have strong antifeedant activity against third-instar larvae of both leaf folders (Ramachandran and Khan, unpublished data). Thus our present study also supports the concept that empirical correlations are possible between the shape of EAG signals elicited by a compound and its behavioral effect on insects.

Synthesis

The olfactory receptor system of the two sympatric leaf folder species is well adapted to perception of a wide range of plant volatiles. Like several other insect species, leaf folder moths have antennae "selectively tuned" to perception of green leaf odor alcohols and aldehydes (Visser, 1983, 1986). However, unlike the other insect species, where response was selective for C_6 alcohols and aldehydes, leaf folders appear to be more sensitive to C_9 and C_6 aldehydes. Aside from compounds of the green leaf volatile complex, moths also were found to respond well to terpene compounds such as α-terpineol, (−)-myrtenal, and citronellal. Whether these compounds are present in rice and other host plants of leaf folder is being currently investigated. We hope that this information, in conjunction with ongoing behavioral experiments, would help in utilizing these compounds in rice pest management.

Similarity in the volatile profiles of both species probably reflects the common host plants of these two species. The limited range of chemicals to which the relatively oligophagous *M. patnalis* was found to respond more than the polyphagous *C. medinalis* may be a reflection of the diet breadth of the two species. However, without information on the distribution of these chemicals in graminae and the behavioral reactions of the two species to these chemicals, this conclusion is premature. More differences were apparent between the sexes than between the species. Males of both species tended to respond more to compounds of the green leaf odor complex, while responses of females for terpene compounds were higher. It is suggested, therefore, that the general green leaf volatiles might be used by both sexes to locate the host habitat. Females, on reaching the host habitat, might continue to use more host-specific volatile compounds such as terpenes for locating suitable oviposition sites. This view supports that of Hansson et al. (1989), who postulate that when pheromone communication started evolving in insects, the sensilla of males sensitive to some of the host-plant volatiles evolved to pheromone receptors with a greatly increased sensitivity to pheromone components emitted by the females.

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CHROMATOGRAPHIC ANALYSIS OF SPECIES SPECIFIC ODOR PROFILES IN *Mastomys natalensis* AND *M. coucha* (RODENTIA: MURIDAE)

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Abstract—Whole-body volatiles from males of the cryptic multimammate mouse species *Mastomys natalensis* and *M. coucha* were analyzed by dynamic solvent effect sampling and capillary gas chromatography. One compound, 3-nonene-2-one, was always present, sometimes as the major component, in volatiles from *M. coucha* and absent, or present only at low levels, in volatiles from *M. natalensis*. The mean ±SD of the 3-nonen-2-one peak area for *M. coucha* was 8599 ± 9630 and for *M. natalensis* 148 ± 486. Chromatographic analysis was more reliable in identifying a male's species than were a female's in a two-choice olfactorium.

Key Words—Multimammate mice, *Mastomys coucha*, *Mastomys natalensis*, dynamic solvent effect, capillary chromatography, odor, 3-nonen-2-one, speciation, cryptic species.

INTRODUCTION

Recognition of, and preference for, conspecific odors is widespread among rodents. A preference for the odor of conspecifics over that of closely related species has been demonstrated in *Mus musculus* (Bowers and Alexander, 1967),

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Peromyscus spp. (Moore, 1965; Doty, 1972, 1973; Drickamer, 1984), *Cavia* spp. (Beauchamp et al., 1979), *Meriones* spp. (Dagg and Windsor, 1971), *Apodemus sylvaticus* (Stoddart and Smith, 1984), *Microtus* spp. (De Jonge, 1980) and *Mastomys* spp. (Apps et al., submitted). Similar discrimination occurs at the subspecies level in *Spalax ehrenbergi* (Nevo et al., 1976) and *Clethrionomys* (Godfrey, 1958).

On this evidence semiochemicals are potentially important contributors to the recognition of conspecific mates and to the maintenance of species' gene pool limits. Two findings are of particular relevance here: in *Peromyscus californicus* and *P. eremicus* only mice from sympatric populations prefer the odor of their own species over that of the other (Carter and Brand, 1986), and in house mice (*Mus musculus*) odor preferences contribute to ethological isolation between adjacent demes (Cox, 1984).

The demonstration of odor preferences is a straightforward and nonetheless powerful technique, but it suffers from the disadvantage that the odor differences between individuals can be demonstrated only by the use of other animals. This introduces a number of variables, e.g., reproductive condition, learning, diet, and social status, which may not be rigorously controllable, may covary with the odor difference, and may themselves be relevant to mate recognition.

An instrumental technique for odor analysis can yield data that is rigorously quantitative and independent of the interfering variables affecting the preferences of test animals. Chemical identification of relevant compounds for bioassay is also possible. The aim of this investigation was to establish the validity of such an analytical approach by studying odor differences between the cryptic multimammate mouse species *Mastomys natalensis* and *Mastomys coucha*, using dynamic solvent effect sampling and gas chromatographic analysis of volatiles from individuals of each species.

Mastomys natalensis and *M. coucha* are nocturnal murids, remarkable for their high reproductive rates, and of considerable medicozoological importance in the biology of bubonic plague and as a disease model (Green et al., 1978; Keogh and Price, 1981; Bronner et al., 1988). Although indistinguishable on external morphology, the two species differ in karyotype (*M. coucha* $2N = 36$, *M. natalensis* $2N = 32$), chromosome banding pattern, hemoglobin electromorphs, courtship behavior, and sperm morphology (Lyons et al., 1977, 1980; Gordon, 1978, 1984; Green et al., 1980; Smithers, 1983, pp. 253–256; Gordon and Watson, 1986). *Mastomys coucha* tends to occur in drier regions (<600 mm isohyet) of southern Africa than *M. natalensis*, although many areas of sympatry have been found (Gordon, 1984). Viable, but infertile, hybrids result when interbred in captivity, but no hybrids have been detected in natural populations (Green et al., 1980).

Species-specific olfactory preferences have been demonstrated in *Masto-*

mys, and although both species show conspecific odor preferences, *M. coucha* is much less discriminating than *M. natalensis* (Apps et al., submitted). Whether this asymmetry is a property of the chemical signal or of the receiver's responses provided a model problem for the analytical approach.

METHODS AND MATERIALS

Whole-body odors of live mice were sampled by dynamic solvent effect (Apps et al., 1987). Each mouse was transferred from its home cage to a 500-cm^3, wide-necked glass jar and charcoal-filtered air was blown through the jar, over the mouse, and through a dynamic solvent effect concentrator at a flow rate of 20 cm^3/min for 20 min (Figure 1). *n*-Hexane was used as solvent in the concentrator. Condensed water was evaporated from the concentrator by a 10 cm^3/min flow of palladium cell purified hydrogen for 15 min. Volatiles accumulated from the mouse's odor were quantitatively analyzed by high-resolution gas–liquid chromatography using a Varian 3700 gas chromatograph fitted with a dynamic solvent effect inlet (Apps et al., 1987). The column was 25 m × 0.3 mm × 0.4 μm methyl silicone produced in house, with hydrogen as carrier gas at a linear velocity of 50 cm/sec. Detection was by FID at a sensitivity of 10^{-11} A/mV, and the signal was fed to a Varian 4270 integrator with a full-scale deflection of 1 mV. The starting temperature was 40°C for inlet and column; the inlet was heated ballistically to 220°C after 2.2 min, and the column was

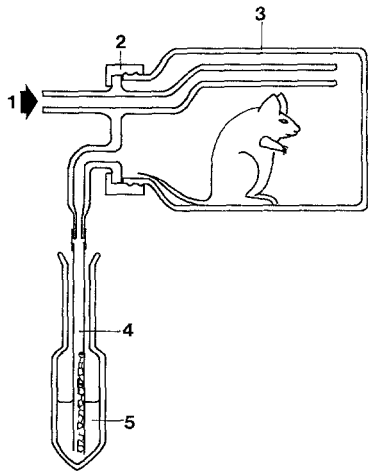


FIG. 1. Apparatus used to sample whole-body volatiles from *Mastomys*. 1: 20 cm^3 min charcoal-filtered air, 2: screw cap, 3: 500 cm^3 glass jar, 4: dynamic solvent effect concentrator, 5: *n*-hexane solvent.

temperature programmed at 5°C/min after 6 min. A blank of the complete analytical and sampling system was run every day.

A total of 17 adult male mice (six *M. coucha*, ten *M. natalensis* and one hybrid) were used as odor donors. Most of the donors were sampled three times; one of the *M. coucha* was sampled four times, and six of the *M. natalensis* were sampled twice each. At least one day elapsed between successive samples from the same animal. All the mice originated from colonies maintained at the Transvaal Museum (TM) and the South African Institute for Medical Research (SAIMR). Specific identity of each individual was confirmed by noting the species diagnostic hemoglobin electromorph (Green et al., 1978) resolved by starch gel electrophoresis of whole blood on a Tris–EDTA–borate buffer system (Selander et al., 1971). The TM animals were housed separately; those from the SAIMR were housed in an all-male group. All animals were supplied with rat pellets and water ad libitum.

The series of chromatograms was examined for peaks whose areas differed consistently between, but not within, species. The compound represented by the peak that most nearly fulfilled this criterion was identified by gas chromatography–mass spectrometry using a Varian 1400 chromatograph with open split interface to a VG Micromass 16F mass spectrometer operated in the electron impact mode. The electron energy was 70 eV, and the source temperature was 200°C. Analytical conditions were equivalent to those above except that the sampling time was extended to 40 min and the chromatographic carrier gas was helium.

RESULTS

The whole-body odors of both species yielded very complex chromatograms after high-resolution separation (Figure 2). Inspection of the chromatograms revealed only one peak, arrowed in Figure 2, whose area was species specific. This peak was absent from, or small in, chromatograms of *M. natalensis* volatiles and was always present, sometimes as the major component, in chromatograms of *M. coucha* volatiles. The overlap between *M. coucha* and *M. natalensis* in the area of this peak was small and was reduced still further if its percentage contribution to the total peak area was considered (Figure 3). The mean and standard deviation of the peak area for *M. natalensis* was 148 ± 486 and for *M. coucha* 8599 ± 9630. The peak was absent from chromatograms of the odor of the single hybrid. Since, for both species, the distribution of peak areas was heavily skewed, the significance of the difference between the species was tested by the Mann-Whitney U test using the mean peak area for each individual. The interspecific difference was significant at the 0.1% level. The species-specific compound was identified as 3-nonen-2-one on the basis of mass spectral comparison with library material.

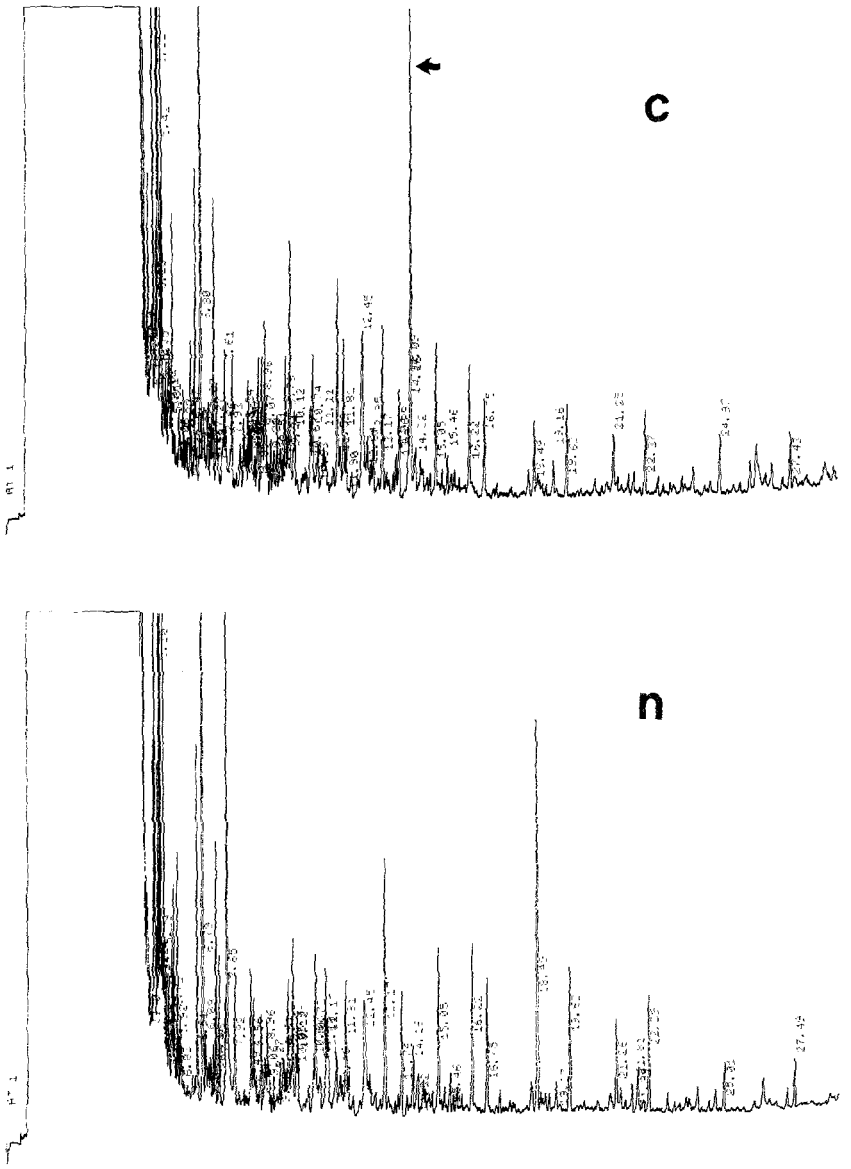


FIG. 2. Chromatograms of whole-body volatiles from a male *Mastomys coucha* (c) and a male *M. natalensis* (n). Volatiles were collected by dynamic solvent effect for 20 min at a flow rate of 20 cm^3/min and separated on a 25-m methylsilicone capillary column with a temperature program of 5°C/min. Detection was by FID with a sensitivity of 1×10^{-11} A/mV full-scale deflection. The peak due to the species-specific compound 3-nonen-2-one is indicated by an arrow.

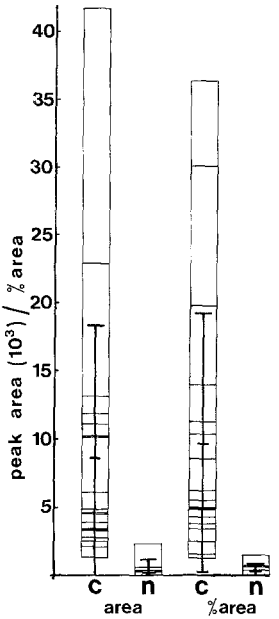


FIG. 3. Areas and percentage areas of 3-nonen-2-one peaks in chromatograms of whole-body volatiles from male *Mastomys coucha* (c) (N = 19) and *M. natalensis* (n) (N = 24). The heavy vertical bar spans one standard deviation either side of the mean (truncated at zero); the light horizontal bars are actual values from each sample, and the light vertical bars span the range of actual values. In 20 samples from *M. natalensis* the peak area was zero.

DISCUSSION

While other investigators of the chemistry of rodent semiochemicals have restricted their attention to either urine (Liebich et al., 1977; Schwende et al., 1984; Novotny et al., 1986) or glandular secretions (Stoddart, 1977), we avoided the problem of prejudging the source of the species-specific odors in *Mastomys* by sampling the whole-body odors of live mice.

The possibility that the observed differences in chromatographic profile are due only to analytical variation must be acknowledged. Nevertheless, neither the magnitude nor the type of differences encountered can be considered likely to have arisen from this source. Dynamic solvent effect sampling has been shown to yield coefficients of variation of better than 10% for nanogram quantities of gas-phase volatiles (Apps, 1988), whereas the differences considered here range over three orders of magnitude for 0- to 21-ng quantities.

The area of the 3-nonen-2-one peak is a measure of that compound's con-

centration in the air around the odor donor but should not be assumed to be related directly to the odor that would be perceived by another rodent. In the following discussion we assume only that perceived odor and chromatographic odor profile are ordinally related.

The quantitative chemistry of the species-specific odor is consistent with the results of odor preference tests (Apps et al., submitted). Female *M. natalensis* show a stronger preference for conspecific male odor than do female *M. coucha*. If the scatter of odor intensities generated by the males of each species reflects the range of odor intensities over which a male is recognized as conspecific by a female, then the overlap between the recognition range of female *M. coucha* and the odor range of male *M. natalensis* is much greater than the corresponding overlap for female *M. natalensis* and male *M. coucha*. Thus, a male *M. coucha* is more likely to fall outside the conspecific recognition range of a female *M. natalensis* than is a male *M. natalensis* to fall outside the conspecific recognition range of a female *M. coucha* (Figure 3). This asymmetry in the properties of the odor signal is sufficient to account for the asymmetry in the females' odor preferences.

That such a small chemical difference should be associated with species-specific odor preferences is, at first sight, surprising. Nevertheless, in extracts of rodent secretions, Stoddart (1977) also found interspecific differences to be small compared to intergeneric, sexual, and age differences.

The absence from all *M. natalensis* chromatograms of any other peak as large as that for 3-nonen-2-one in the *M. coucha* chromatograms and the occasional presence of 3-nonen-2-one in *M. natalensis* demonstrates that the difference between the species is quantitative rather than qualitative. In the latter case the occurrence of a compound unique to *M. natalensis*, and possibly chemically related to 3-nonen-2-one, would be expected.

Although the precise origin of the odor difference was not investigated, environmental influences can be ruled out since all subjects received the same diet and were housed under uniform conditions. Because both social status (Apps et al., 1988) and reproductive condition (Jemiolo et al., 1987) have analytically detectable effects on the odors of laboratory mice, only adult males that, except for those from SAIMR, were individually housed were used in this investigation. Thus, some aspect of odor production presumably is under genetic control and, therefore, could contribute to maintenance of species gene pools. In house mice single gene differences at the *T* and the H_2 loci affect not only odor but also odor preference (Yamazaki et al., 1980; Lenington, 1983; Drickamer and Lenington, 1987) and in the H_2 case the odor differences are analytically detectable (Schwende et al., 1984). In both these cases the odor differences form the basis of selection of mates carrying "good genes."

The interpretation of the significance of the wide scatter of peak areas within each species is complicated by an almost equally wide range of peak

areas in replicate samples from given individuals. Among *M. coucha*, the two highest and the third lowest levels of 3-nonen-2-one were produced by one individual, and among *M. natalensis* one individual produced both the highest and the equal lowest levels. While such fluctuations in odor emission may be under voluntary control, it seems more likely that they are related to episodic changes in hormone levels; quantitative fluctuations in odor also are found in male laboratory mice (Apps et al., 1988) where testosterone secretion is known to be pulsatile (Bartke and Dalterio, 1975). What the consequences of odor fluctuations are for recognition of conspecifics will depend to a large extent on the nature of the discrimination effected by the females. If, for example, the discrimination is based on whether the contribution of 3-nonen-2-one to the male's odor exceeds a certain threshhold, then differences, both within and between individuals, almost as wide as those recorded here may be of no significance.

Since the principal aim of this study was to examine the validity of an instrumental, analytical approach to problems of mammalian species-specific odor signals, it is of interest to compare the performance of the analytical system with that of *Mastomys* females presented with odors from males of both species in a two-choice olfactorium. Of seven female *M. coucha*, five sniffed more at the odor of a homospecific male, but in only three cases was the difference significant ($P < 0.05$). Of four female *M. natalensis* all sniffed more at male *M. natalensis* odor and three of these differences were significant (unpublished). If those 3-nonen-2-one peak areas for one species that fall within the range of peak areas for the other species are taken as cases of incorrect identification, then two of 19 samples from *M. coucha* and one of 24 samples from *M. natalensis* were misidentified. If the "true" odor of each individual is taken as the mean of its sample peak areas, then no individuals of either species were misidentified. This brings us to the rather surprising, but nonetheless encouraging, conclusion that odor analysis by dynamic solvent effect sampling and capillary gas chromatography is actually more reliable in recognizing to which species a *Mastomys* male belongs than are conspecific females in a two-choice olfactorium.

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BIOCHEMICAL ACTIVITY OF CENTIPEDEGRASS AGAINST FALL ARMYWORM LARVAE

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Abstract—Centipedegrass, *Eremochloa ophiuroides* (Munro) Hack, severely inhibits growth of the fall armyworm larva, *Spodoptera frugiperda* (J.E. Smith). Fresh centipedegrass extracts and extract fractions were deposited on Celufil, incorporated into meridic-based diets and bioassayed against neonate larvae of the fall armyworm in the laboratory. The methanol extract (F_1) caused the greatest reduction in larval weight. When F_1 was partitioned between methylene chloride and water, the activity was transferred to the water-soluble fraction (F_5), which, when further fractionated using preparative C-18 reverse-phase chromatography, yielded active F_7 and F_8 fractions. Gas chromatograph–mass spectrometry and high-pressure liquid chromatography (HPLC) showed F_7 to be 95% caffeoylquinic acids with chlorogenic acid as the major constituent. HPLC analysis of F_8 revealed maysin [2″-*O*-α-L-rhamnosyl-6-*C*-(6-deoxy-*xylo*-hexos-4-ulosyl)luteolin] and other luteolin derivatives. Chlorogenic acid and other caffeoylquinic acids, maysin, and other luteolin derivatives are the major factors responsible for the antibiotic resistance of centipedegrass to larvae of the fall armyworm.

Key Words—Resistance, *Spodoptera frugiperda*, Lepidoptera, Noctuidae, antibiosis, bioactivity, plant biochemicals, chlorogenic acid, maysin, luteolin, fall armyworm, centipedegrass, *Spodoptera frugiperda* (J.E. Smith).

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INTRODUCTION

The fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith), is one of the most destructive pests in the southeastern United States because of its high mobility and polyphagy and its refractiveness to management (Luginbill, 1928). It severely damages not only important food and fiber crops, but also damages grasses grown for pasture, silage, turf, or soil stabilization (Kelsheimer et al., 1953).

Plant resistance to insect pests has been reported as "the most effective and ideal method of combating insects that attack crop plants" (Luginbill, 1969). Very little information was available on resistance of grasses to FAW until recently (Wiseman and Davis, 1979). Wiseman et al. (1982) demonstrated a high level of both antibiosis (growth inhibition) and nonpreference resistance to the fall armyworm in common centipedegrass, *Eremochloa ophiuroides* (Munro) Hack. The weight of FAW larvae feeding on fresh centipedegrass was reduced by 80–99% (Wiseman et al., 1982). Chang et al. (1985a, b, 1986) also studied the effect of centipedegrass on behavior, growth, and development of FAW larvae and found (Chang et al., 1985b) that the preference of FAW for centipedegrass was increased by ninefold and damage by 16-fold when nitrogen (N) fertilizer was applied once every two weeks. However, centipedegrass is normally fertilized with very little N, while bermudagrass is fertilized with 100–450 kg N/ha. Under normal turf management, common centipedegrass is highly resistant to the FAW while common bermudagrass is susceptible.

However, laboratory assays with lyophilized centipedegrass or common bermudagrass incorporated into a pinto bean diet decreased the weight of larvae by only 25–30% for larvae that were fed on centipedegrass compared with those that were fed on bermudagrass (Wiseman et al., 1986). Gueldner et al. (1986) postulated that antibiotic resistance factors present in fresh centipedegrass were plant polyphenols that are rapidly degraded when fresh or freeze-dried centipedegrass is incorporated into bioassay diets.

In this paper we report the isolation and identification of major chemicals contributing to the antibiotic resistance of centipedegrass to larvae of the FAW.

METHODS AND MATERIALS

Materials. Centipedegrass was harvested from an unfertilized lawn in May and July of 1987. Leaf grass blades were clipped with scissors and either immediately (1) homogenized in methanol (MeOH) with a Waring blender or (2) frozen and later freeze-dried. The homogenized samples of freshly clipped grass were placed in brown solvent jars, cooled with Dry Ice, and stored at $-17°C$ until fractionated as shown in Figure 1. Freeze-dried centipedegrass was ground

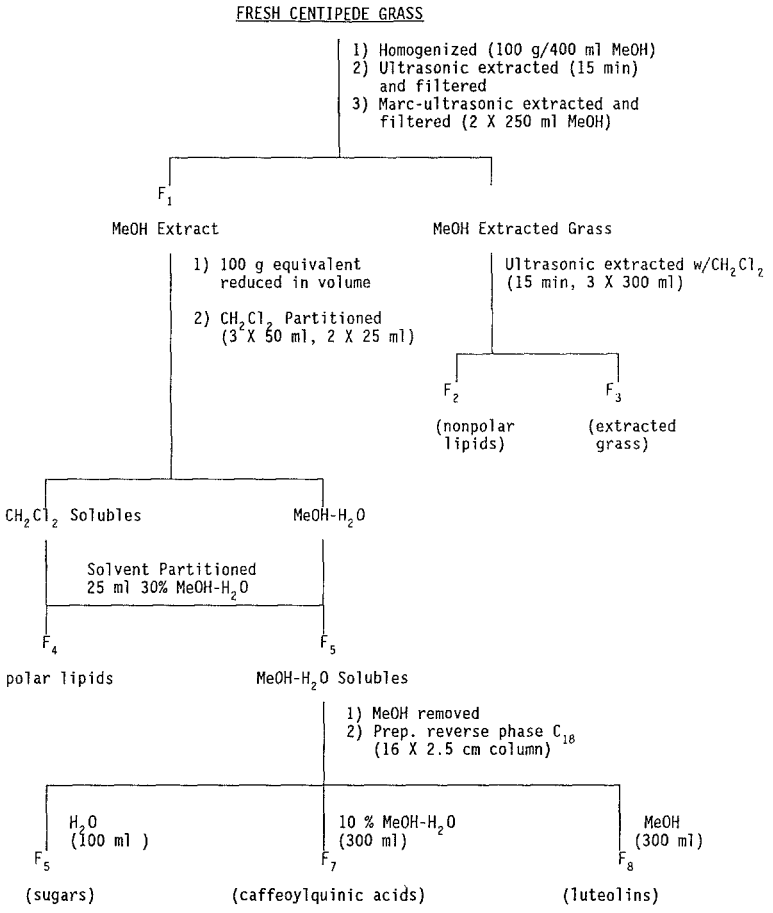


FIG. 1. Extraction and fractionation scheme for the isolation of compounds responsible for antibiosis in fresh centipedegrass against the FAW.

in a Wiley mill, stored at −17°C, and also fractionated as shown in Figure 1. After the samples had warmed to room temperature, the jars were placed in an ultrasonic bath and sonicated for 15 min. The extraction processes were repeated twice, with MeOH being removed by filtration after each extraction. MeOH extracts were then combined to yield fraction 1 (F_1). The MeOH extracted grass was dried in a vacuum desiccator at room temperature and then ultrasonically extracted with methylene chloride (CH_2Cl_2) to yield fraction 2 (F_2). The MeOH–CH_2Cl_2-extracted grass was dried under vacuum to yield fraction 3 (F_3).

A 100-g fresh weight equivalent of F_1 was reduced to about 150 ml volume on a rotoevaporator at 40°C and 15 mm Hg and partitioned against CH_2Cl_2.

The F_1 CH_2Cl_2 solubles were combined and washed with 30% MeOH–H_2O to yield fraction 4 (F_4). The MeOH–H_2O fractions were combined to yield fraction 5 (F_5).

A 50-g fresh weight grass equivalent of F_5 was reduced to about 40 ml volume on a rotoevaporator and placed on a preparative low-pressure C_{18} reverse-phase column and eluted with 100 ml H_2O to yield fraction 6 (F_6), with 300 ml of 10% MeOH–H_2O to yield fraction 7 (F_7), and finally eluted with 300 ml of MeOH to yield fraction 8 (F_8).

The fractions and other components for biological bioassays were dissolved in MeOH and were deposited on 5 g for Celufil (U.S. Biochemical, Cleveland, Ohio) (1× rate = 50 g fresh weight, 0.5× = 25 g fresh weight, and 0.25× = 12.5 g fresh weight equivalents) in a round-bottom flask on a rotoevaporator as described earlier. Deposited samples were transferred to vials and placed under oil pump vacuum for 3 hr and then stored at −10°C until incorporation into the bioassay medium. A solvent Celufil blank was prepared for each test.

All solvents were Burdick and Jackson distilled in glass grade; NO-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and dimethylformamide (DMF) were obtained from Pierce Chemical Company. Chlorogenic acid was obtained from Aldrich Chemical Company. Maysin was isolated from corn silks (Snook et al., 1989).

Bioassay. Neonate larvae of the FAW and the pinto bean diet (Perkins, 1979) were obtained from the insect-rearing services of the Insect Biology and Population Management Research Laboratory. Pinto bean diet was diluted at the ratio of 7 ml diet to 3 ml distilled water, blended thoroughly, and held in a hot water bath at 45°C. Centipedegrass fractions were deposited on 5 g of Celufil, mixed into the 50 g of diluted bean diet, and thoroughly mixed in a microblender for ca. 20–30 sec. An untreated pinto bean check diet and Celufil check diet also were included for comparisons. The diet mixtures were each poured into a large syringe (60 cc), dispensed into seven to eight plastic soda straws (ca. 20 cm in length and 6 mm ID), and allowed to solidify for ca. 2 hr. Then the straws were cut on a 45° angle into 72 2-cm sections. Two sections were placed in each of 36 18-mm plastic diet cups with one neonate FAW larva and capped with a polycoated lid. The use of the small cup and the polycoated lid prevented loss of moisture from the micro-diets (Wiseman et al., 1986). The experimental design for each test was a randomized complete block having 18 replications with two cups per replicate. Insect feeding trials were conducted in an environmental room operated at 26.7°C, 75–80% relative humidity, and a 14 : 10 hr photoperiod. The weight of each larva was recorded seven and nine days after feeding began (Wiseman et al., 1984).

Commercially available chlorogenic acid was deposited on Celufil at a volume of 0, 37.0, 75.0, 125.1, 249.5, or 499.4 ml. The mixture was then incor-

porated into 50 g diluted pinto bean diet and bioassayed against FAW larvae using the experimental design and procedures described above.

Data for the weight of test larvae computed as a percent of the weight of FAW on the Celufil blank (=100) were analyzed by an ANOVA (SAS Institute, 1985). Significantly different means were separated by Waller-Duncan k ratio t test, with a k ratio = 100 and $P \leq 0.05$ (Waller and Duncan, 1969).

Chromatographic Analysis. Portions of the centipedegrass fractions were transferred to micro-autosampler vials and desiccated to dryness under a stream of N_2 at 40°C. Fifty microliters of 1:1 BSTFA–DMF was added, vials were then capped, placed in an ultrasonic bath for 15 min, and heated for 45 min at 75°C. The samples were analyzed by capillary gas chromatography on an HP5890 gas chromatograph (0.54 mm ID × 12 m bonded SE-S4 fused silica column, 20 ml/min, H carrier gas, temperature program of 100°C/2 min to 310°C at 7°/min; 310°C for 10 min; splitless injection, purge activation time 2 min).

High-pressure liquid chromatograms in Figure 2 were obtained using a Waters Radial-Pak, 5-μm, C-18 reverse-phase cartridge for the RCM-100 radial

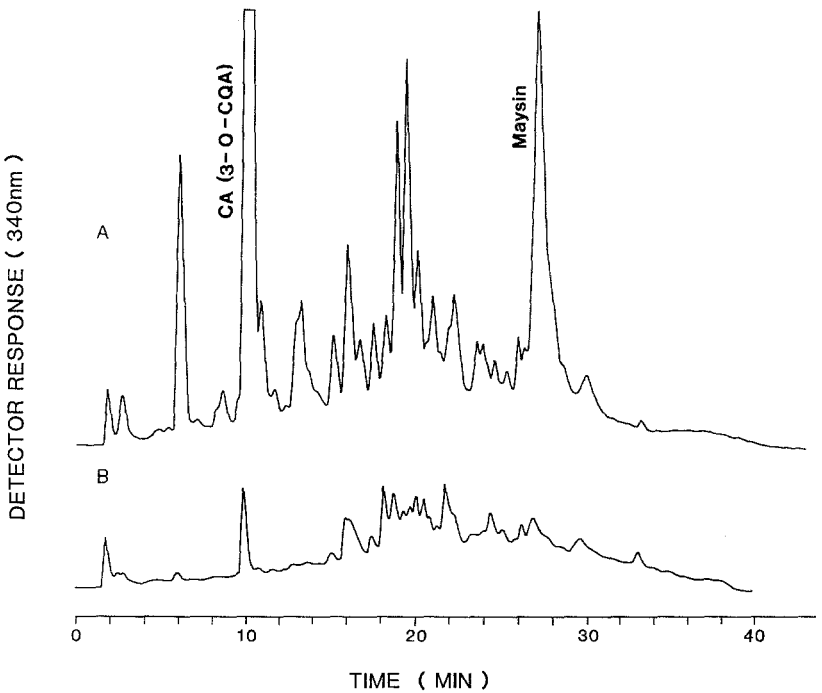


FIG. 2. HPLC chromatogram of freeze-dried centipedegrass immediately after rehydration (A) and 1 hr after rehydration (B).

compression module and a Waters model ALC-402 liquid chromatograph equipped with a Waters model 680 gradient controller, a Perkin–Elmer model LC-55 variable wave length UV/VFS detector, and a Shimadzu Chromatopac C-R3A integrator. MeOH and water containing 2% acetic acid were used as eluents in a gradient of 5:95 MeOH–water to 60:40 MeOH–water in 30 min. A flow rate of 1 ml/min was used, and the eluent was monitored at 340 nm.

Fractions F_1, F_5, F_7, and F_8 also were analyzed using a Hewlett-Packard model 1090 liquid chromatograph equipped with a model 1040 diode array UV/VFS detector and data system, and a 4.6-mm × 25-cm, 5-μm, C-18 reverse-phase column maintained at 45°C. Eluting solvents were MeOH and water each containing 0.5% phosphoric acid at a flow rate of 0.8 ml/min. A gradient of 20% MeOH to 30% MeOH in 5 min, 45% in 20 min, 50% in 21 min, 60% in 25 min, and 100% in 40 min was used and elution was monitored at 340 nm.

Component Characterization. The presence of chlorogenic acid and the other caffeoylquinic acids and maysin were confirmed by comparing HPLC retention and HPLC/UV data obtained using the diode array detector with authentic standards. The other luteolin derivatives were characterized only by HPLC UV data obtained with the diode array detector. Sucrose and the caffeoylquinic acids were also characterized by comparing their GC retention and GC-MS data of their silylethers with those of standard compounds. GC-MS data were obtained using a HP5985B GC-MS system modified for capillary GC-MS as described by Arrendale et al. (1984).

RESULTS

Extraction, Fractionation and Characterization of Centipedegrass Components. When centipedegrass was mixed with the meridic diet, a brown layer consisting of polyphenols formed on top of the diet (Wiseman et al., 1986; Chang et al., 1985b). HPLC of the polyphenol extract, obtained immediately after rehydration of freeze-dried centipedegrass is shown in Figure 2A. Based on the total peak area (Figure 2B), over 97% of the components were destroyed within 1 hr after rehydration. These observations from previous studies indicate that the antibiotic factors in centipedegrass were being oxidized in the bioassay diet, possibly by oxidative enzymes present in the grass, and that deactivation of the oxidation systems was necessary before quantitative isolation of the antibiotic factors could be achieved.

The results of the initial bioassay (Table 1) based on seven- and nine-day weight of FAW larvae showed that the chemicals responsible for antibiosis to the FAW larvae in centipedegrass were concentrated in the F_1 and F_5 fractions. Gas chromatograms of F_1 and F_5 fractions obtained after silylation [free hydroxyl and acid moieties converted to trimethylsilyl (TMS) derivatives] are compared in Figure 3, and an HPLC chromatogram of F_1 is shown in Figure 4. The HPLC

TABLE 1. RELATIVE WEIGHT OF FALL ARMYWORM LARVAE 7 AND 9 DAYS AFTER FEEDING ON FRESH CENTIPEDEGRASS EXTRACTS OR FREEZE-DRIED CENTIPEDEGRASS

Treatment	Rate	Relative larval weight (%)[a]	
		7 day	9 day
Bean diet (BD)		131a	118a
BD + F_1 (MeOH extract)	1.00X	2h	1h
	0.50X	12fgh	8gh
	0.25X	24f	20g
BD + F_2 (nonpolar lipids)	1.00X	92b	94bcd
	0.50X	92b	100bc
	0.25X	96b	97bcd
BD + F_3 (extracted grass)	1.00X	94b	86cde
	0.50X	88bc	86cde
	0.25X	104b	101b
BD + F_4 (polar lipids)	1.00X	55e	58f
	0.50X	74cd	76e
	0.25X	76c	80de
BD + F_5 (MeOH–H_2O-soluble)	1.00X	6g	3h
	0.50X	12fgh	9gh
	0.25X	24f	21g
Centipedegrass (freeze-dried)	1.00X	73cd	54f
	0.50X	74cd	56f
	0.25X	73cd	54f

[a] Percent relative larval weight = mean larval weight treatment/mean larval weight Celufil blank × 100. Relative weight means within a column not followed by the same letter are significantly different ($P \leq 0.05$) (Waller and Duncan, 1969). 1× = 50g fresh weight centipedegrass equivalent.

analysis of the F_5 fractions were essentially identical to the analysis results of the F_1 fraction. GC data from the various fractions indicated that 95+% of the methylene chloride soluble components in F_1 were transferred to F_4. HPLC/UV data obtained using a diode array detector revealed a series of caffeic acid derivatives (caffeoylquinic acids) and luteolin derivatives in F_5. The major components of each type of caffeic acid were chlorogenic acid (3-*O*-caffeoylquinic acid) and maysin [2″-*O*-α-L-rhamnosyl-6-*C*-(6-deoxy-*xylo*-hexos-4-ulosyl)luteolin]. Major components, characterized by retention time and GC-MS data, were an early-eluted unknown polyhydroxy compound, sucrose, and chlorogenic acid. The luteolin compounds did not form stable GC-volatile TMS derivatives.

For the next insect bioassay (Table 2), a preparative C-18 reverse-phase chromatographic system was used to separate F_5 into a sugar fraction (F_6), a chlorogenic acid and other caffeoylquinic acids fraction (F_7), and a luteolin

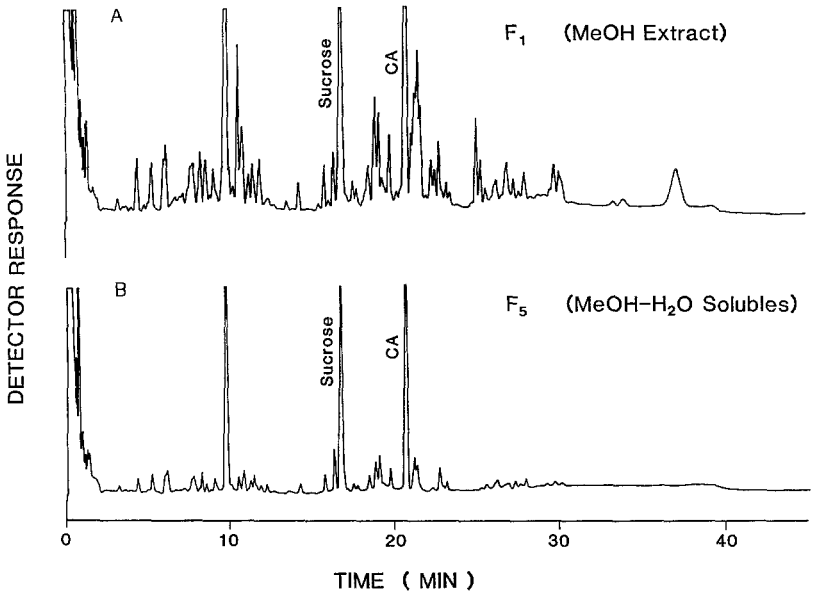


FIG. 3. Gas chromatograms of fractions F_1 (A) and F_5 (B) after conversion of hydroxylated components to trimethylsilyl ethers.

fraction (F_8). Gas chromatography (Figure 5) and HPLC indicated that F_7 was 95+% caffeoylquinic acid isomers with chlorogenic acid being the major constituent. The HPLC chromatogram of F_8 indicated it was greater than 95% luteolin derivatives. Less than 5% of the caffeoylquinic acid compounds in F_5 were present in F_8.

Bioassay. The weight of seven- and nine-day larvae showed that the majority of the antibiotic resistance factor(s) were from extracts of fresh grass extracted by MeOH (F_1) (Table 1). The weight of larvae feeding on the nonpolar lipid fraction (F_2) and the MeOH and CH_2Cl_2 extracted grass (F_3) was significantly lower than the weight of larvae that fed on the diet with the Celufil blank (check). Except for the seven-day 1× rate (the MeOH–H_2O-soluble fraction from F_1) treaments did not significantly reduce the weight of larvae from that of the F_5 fraction below weight of larvae that fed on F_1.

Weight of larvae that fed on F_1 and F_5 (Table 2) was comparable to the weight of larvae that fed on the same fractions in the first bioassay (Table 1). The weight of larvae feeding on the sugar fraction (F_6) did not have a significantly lower weight from those that were fed the check diets. The antibiotic resistance factor(s) in F_5 was divided between F_7 and F_8. No significant differences in weights were found among larvae that fed on F_7, F_8, F_5, and F_1.

Bioassaying chlorogenic acid against larvae of the FAW produced a definite dose–response (Table 3); each increase in concentration of chlorogenic acid

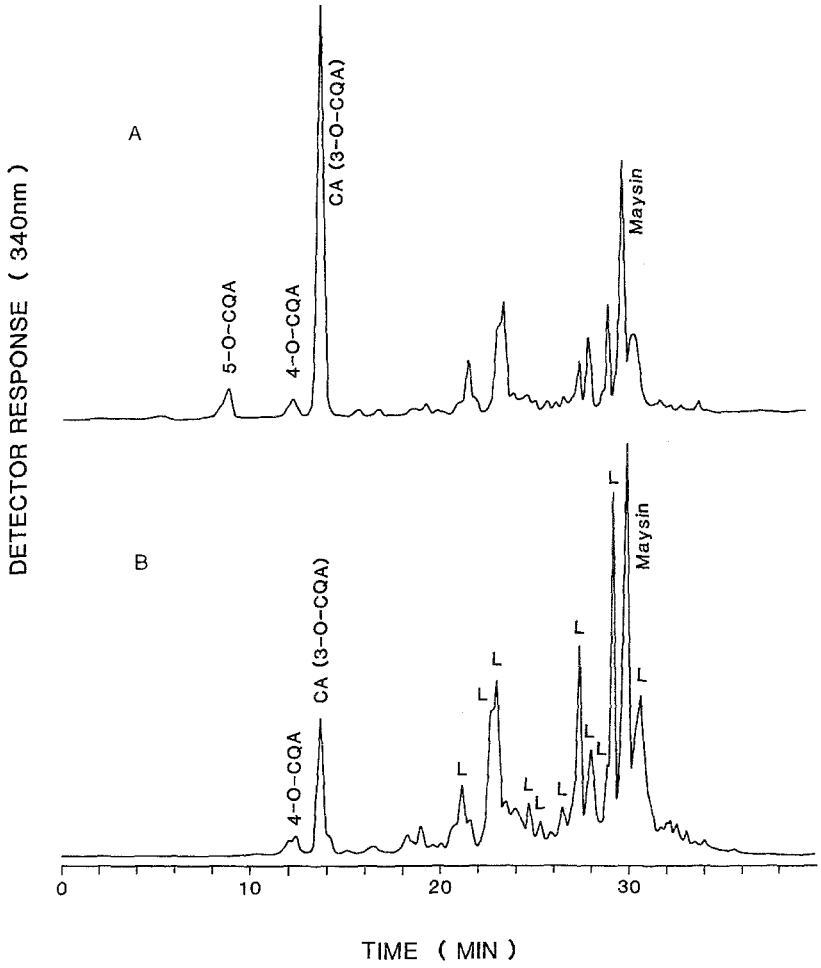


FIG. 4. HPLC chromatograms of fractions F_1 (A) and F_8 (B). Peaks labeled L yielded UV spectra characteristics of luteolin derivatives.

significantly reduced the weight of FAW larvae. The 249.5-mg rate of chlorogenic acid reduced the weight of larvae similar to the reduction in weight of larvae that fed on the 1× rate of F_6 (Table 2).

DISCUSSION

Gueldner et al. (1987) reported that immediate microwave drying and placement of fresh tissue into methanol effectively stopped enzymatic degradation of chlorogenic acid and other polyphenols in fresh tobacco leaves. We

TABLE 2. RELATIVE WEIGHT OF FALL ARMYWORM LARVAE 7 AND 9 DAYS AFTER FEEDING ON 50-g EXTRACTS OF FRESH CENTIPEDEGRASS OR FREEZE-DRIED CENTIPEDEGRASS

Treatment	Relative larval weight (%)[a]	
	7 day	9 day
Bean diet (BD)	92a	104a
BD + F_1 (MeOH extract)	3c	3d
BD + F_5 (MeOH-H_2O-soluble)	7c	4d
BD + F_6 (sugars)	79ab	94ab
BD + F_7 (caffeoylquinic acids)	19c	13d
BD + F_8 (luteolins)	12c	6d
BD + centipedegrass (freeze-dried)	78ab	68c

[a]Relative larval weight = mean larval weight treatment/mean larval weight Celufil blank × 100. Relative weight means within a column not followed by the same letter are significantly different ($P \leq 0.05$) (Waller and Duncan, 1969). Extracts are equivalent to the 50-g fresh weight centipedegrass equivalent.

were able to minimize the degradation of the polyphenolics in freshly-cut centipedegrass by homogenizing the leaves in MeOH. Hence, we were able to obtain laboratory bioassay data for weight reductions of FAW larvae feeding on extracted tissue that was comparable to the weight reduction for FAW larvae that fed on equivalent concentrations of fresh centipedegrass.

Our data make it clear that chlorogenic acid and other caffeoylquinic acids, maysin, and other luteolin derivatives are the major factors responsible for antibiosis to FAW larvae in centipedegrass. Similarly, HPLC analyses of corn silks from a corn line that is resistant to larvae of the FAW also contained chlorogenic acid, maysin, and total luteolins of 0.45, 0.20, and 0.79% fresh weight, respectively (Snook et al., 1989). The F_7 fraction of centipedegrass (Table 2) contained 245 mg of chlorogenic acid; the weight of FAW larvae after feeding on this fraction agreed with the bioassay data obtained with 250 mg of chlorogenic acid (Table 3).

Phenolics occur in many plant species and have been associated with resistance to insects in several species of plants. Phenolics may affect either the preference of the insect for a host plant or impart antibiotic resistance to a pest (Cole, 1985). Chlorogenic acid has been implicated in both resistance and susceptibility in a number of insect–plant relationships. Lindroth and Peterson (1988) found that chlorogenic acid had no deleterious effects on *Spodoptera eridania* (Cramer) when the compound was painted on lima bean leaves on which larvae subsequently fed through the duration of the fifth instar. In addi-

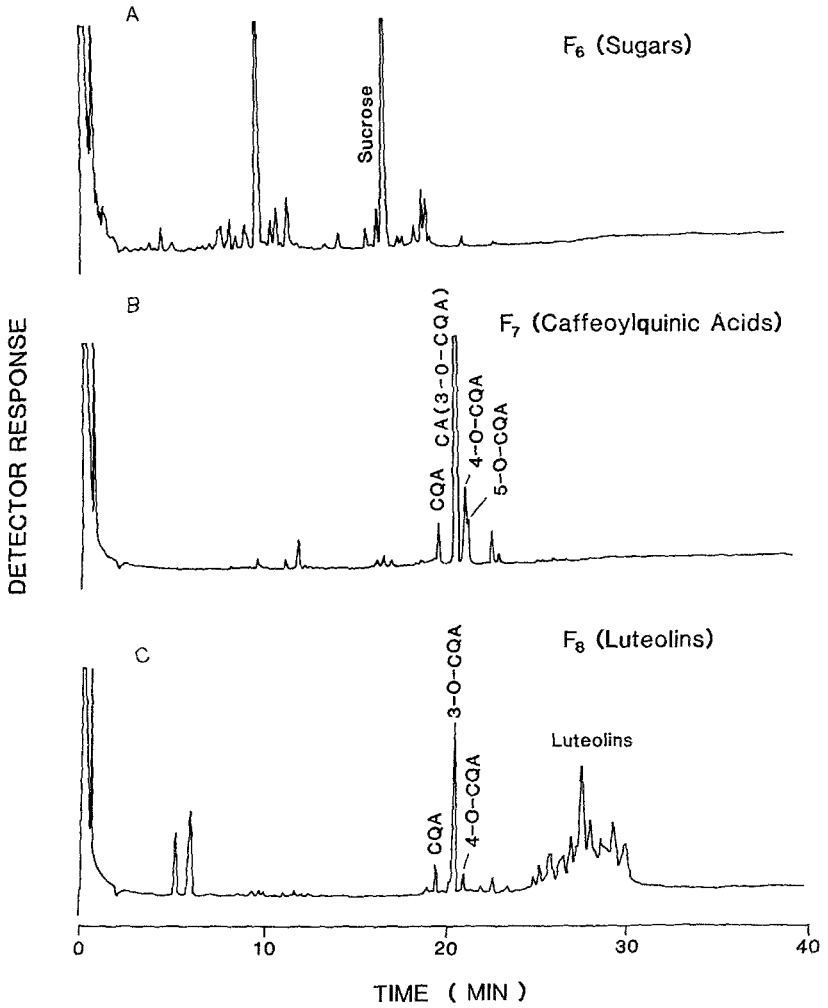


FIG. 5. Gas chromatograms of fractions F_6 (A) and F_7 (B), and F_8 (C) after conversion of hydroxylated components to trimethylsilyl ethers.

tion, Cole (1985) reported that increased concentrations of chlorogenic acid in carrots resulted in greater susceptibility to damage by larvae of the carrot fly, *Psila rosae* (F.). On the contrary, Cole (1984) found a direct correlation between isochlorogenic acid in lettuce and increased resistance of the lettuce to root aphid, *Pemphigus bursarius* (L.). Johnson and Severson (1989) reported that chlorogenic acid depressed the weight of the tobacco budworm, *Heliothis virescens* (F.), larvae similar to the depression of weight for the FAW larvae reported here (Table 3).

TABLE 3. RELATIVE WEIGHT OF FALL ARMYWORM LARVAE 7 AND 9 DAYS AFTER FEEDING ON DIET CONTAINING VARIOUS CONCENTRATIONS OF CHLOROGENIC ACID (CA).

Treatment	Relative larval weight (%)[a]	
	7 day	9 day
Bean diet check	121a	101a
0.0 mg CA (solvent check)	101b	105a
37.0 mg CA	89c	86b
75.0 mg CA	58d	62c
125.1 mg CA	39e	41d
249.5 mg CA	14f	12e
499.4 mg CA	6f	4f

[a]Relative larval weight = mean larval weight treatment/mean larval weight celufil blank × 100. Relative weights within a column not followed by the same letter are significantly different ($P \leq 0.05$) (Waller and Duncan, 1969). 249.5 mg CA equals 1 × rate in Table 2.

Studies on the relationships between maysin and insect development are limited. Wiseman et al. (1977) reported resistance to the corn earworm [*Heliothis zea* (Boddie)] in an exotic corn, Zapalote Chico. Waiss et al. (1979) isolated maysin from the silks of this corn and reported that maysin severely retarded the growth of the corn earworm larva. Recently, Gueldner et al. (1988) proposed that resistance to the FAW in the leaves of teosinte, an ancestor of corn, is due in part to maysin and other luteolin C-glycosides.

Knowledge of the basis of resistance is important in both the development and use of the insect-resistant cultivar. Lawns planted to centipedegrass require less pesticide because of its resistance to the fall armyworm. The use of centipedegrass as a genetic source of resistance to the fall armyworm could reduce the susceptibility of other lawn grasses as well as pasture grasses grown extensively throughout the southeastern United States.

This study is the first that clearly shows the importance of chlorogenic acid and luteolin C-glycosides as natural inhibitory factors limiting growth of the FAW larva. However, at present, the mode of action of these compounds is unknown. This study also suggests a common chemical link of maysin and/or chlorogenic acid among several species of grasses with resistance to insects. As shown in Fig. 4, F_8 is a complex mixture of luteolin derivatives. Based on the studies of Elliger et al. (1980), which showed that free 3′, 4′-dihydroxy groups of flavonoids are important factors in inhibiting growth of corn earworm larvae, we assume that a number of these luteolin compounds are biologically active. In the future, we plan to isolate, characterize, and bioassay a number of these compounds from grasses to verify this assumption.

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DEFENSE CHEMICALS FROM ABDOMINAL GLANDS OF 13 ROVE BEETLE SPECIES OF SUBTRIBE STAPHYLININA (COLEOPTERA: STAPHYLINIDAE, STAPHYLININAE)

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Abstract—Forty-one volatile constituents have been characterized from the abdominal defensive glands of 13 Staphylinina rove beetle species (Staphylinidae). The major secretion component, a rapidly polymerizing iridoid dialdehyde (in most cases iridodial), is mainly accompanied by variable amounts of some iridoid lactones, traces of actinidine, some possible monoterpene precursors, short-chain ketones, cyclic compounds, and spiroacetals. The secretion mixtures even of single Staphylinina genera are very heterogenously composed of a variety of active substances with different structures. This situation may be a typical feature of iridoid defense systems based upon a primary fixative major compound and clearly differs from other chemical defense systems (for example the quinone system) which have a toxic main component.

Key Words—Coleoptera, Staphylinidae, Staphylinina, chemical defense, abdominal glands, iridoids, iridodial, ketones, cyclic compounds, spiroacetals.

INTRODUCTION

The rove beetle subtribe Staphylinina comprises a group of about 45 large staphylinids in Central Europe, which show a very conspicuous defensive

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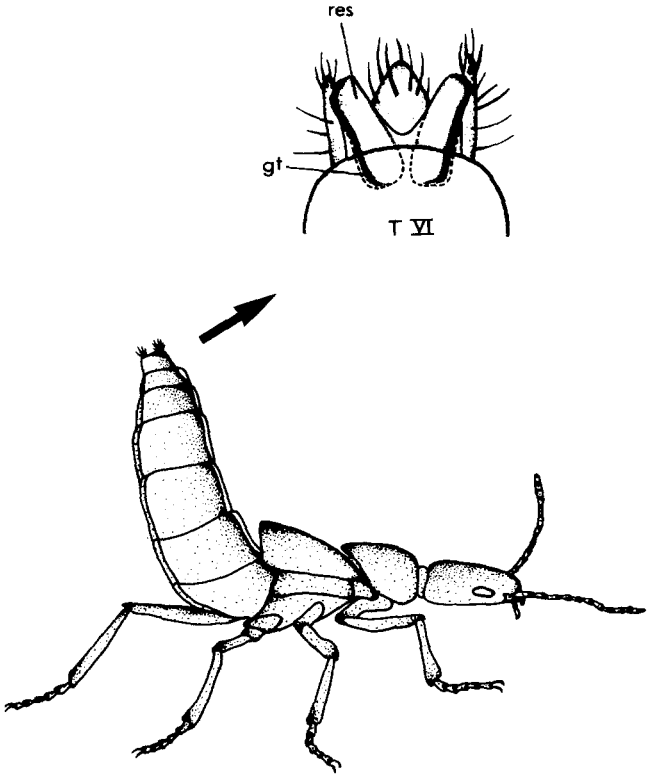


FIG. 1. Lateral view on a specimen of *Ontholestes tesselatus* (Fourcr.) in defensive posture (the abdomen is twisted so that the vetral tergites are turned towards the observer). The arrow points at the dorsal part of the abdominal tip with partly evaginated glands (res: gland reservoir, gt: glandular tissue, T VI: tergite six).

behavior. When molested, all these beetles raise their abdomens and evert a pair of glands that are derived from the membrane between the sixth and seventh apparent abdominal tergite (Figure 1). At the same time a defensive secretion often exudes at the top of the partly everted gland reservoirs and is carefully directed towards the offending agent by the highly maneuverable abdomen.

This obvious usage of the abdominal defensive glands is probably the reason why these structures have been known since at least 1836, when Oken (1836) gave a short description of them in his natural history. A first hint of the participation of a chemical agent in defense of staphylinids was given in 1732 by Linné (see translation, Linné 1975), who observed a large rove beetle (per-

haps a Staphylinina species!) emitting an odor that was comparable to the fragrance of the oil of *Ruta graveolens* L.

Although this early observation suggested the exisistence of a chemical defense mechanism in a large rove beetle, no concrete analysis of the abdominal gland secretion of a Staphylinina species was made until 1971, when Abou-Donia et al. (1971) could demonstrate that the major compound of the *Ocypus olens* secretion was identical with synthetic iridodial. The main volatile of this secretion was identified as 4-methyl-3-hexanone by Fish and Pattenden (1975).

A very detailed investigation was carried out on the chemical defense of *Creophilus maxillosus* (Jefson et al., 1983). The secretion of this species was characterized as a mixture of dihydronepetalactone, iridodial, actinidine, (*E*)-8-oxocitronellylacetate, isoamylalcohol, and isoamylacetate. Moreover, almost all these chemicals were shown to be effective repellents for ants.

The defensive secretion of *Ontholestes murinus* contains, besides iridodial, actinidine, 2-heptanone, and 6-methyl-2-heptanone, some spiroacetals that were found for the first time in a defense gland in this Staphylinina species (Dettner and Schwinger, 1986).

The above mentioned chemical analyses, which have until now been performed only on three members of the Staphylinina subtribe, have revealed an obvious diversity of defensive chemicals, which have for the most part a remarkable biological activity. These results led us to the present investigation, which analyses the defensive chemistry of 13 Staphylinina species and discusses the different chemical patterns with regard to their biological significance.

METHODS AND MATERIALS

Biological Material. The following species of the rove beetle subtribe Staphylinina were collected between June and September 1987 and 1988 near Bayreuth and Tübingen , F.R.G.: *Ontholestes tesselatus* (Fourcr.), *O. murinus* (L.) and *Creophilus maxillosus* L. were caught by hand from dunghills and carrion. *Staphylinus caesareus* Ced., *St. erythropterus* L., *St. dimidiaticornis* Gemm., *Ocypus (Goerius) olens* (Müll.), *O. (Goerius) similis* (F.), *O. (Goerius) ophthalmicus* Scop., *O. (Ocypus) melanarius* (Heer), *O. (Pseudocypous) fuscatus* (Grav.), *O. (Pseudocypus) aenaeocephalus* (Deg.), and *Parabemus fossor* Scop. were mainly collected with small ground traps in miscellaneous habitats, which had been selected according to the faunal descriptions of Horion (1965). The determination and nomenclature followed the key of Lohse (1964). All beetles were transported carefully to the laboratory and immediately frozen.

Chemical Analyses. In order to get some droplets of the defensive secretion, the abdominal glands of a freshly thawed beetle were artificially everted

by exerting a pressure on the abdomen. The fluid exuding at the top of the reservoirs was collected with a microsyringe (Solids Injector, Scientific Glass Engineering, type B) and directly transferred into a capillary gas chromatograph. Mixing of the secretion with fecal fluid, which often spread over the last tergites during the milking process, was carefully avoided, so that all detected chemicals can be regarded as true abdominal gland compounds.

All chemical analyses were carried out as combined gas chromatography–mass spectrometry (GC-MS). studies. The system used consisted of a Carlo Erba GC Vega Series 2 capillary gas chromatograph coupled to a Finnigan MAT Ion Trap Detector ITD 800. As far as possible, GC-MS acquisitions were made using electron impact ionization (EI) as well as chemical ionization (CI) with isobutane as reactant gas. For all GC-MS analyses a 10-m Chrompack WCot FS-CP Wax 52 CB glass capillary column was used. Elution was programmed routinely from 60°C to 170°C with a heating rate of 10°C/min and from 170°C to 230°C with a heating rate of 15°C/min (carrier gas: helium).

RESULTS

The analysis of the defense chemicals of 13 species of the rove beetle subtribe Staphylinina revealed more than 41 secretion compounds. Figure 2 shows representative gas chromatograms of 10 of the beetle species investigated, where the whole bouquet of the abdominal gland secretions could be confirmed in several GC-MS acquisitions. The numbers given to the peaks in the chromatograms refer to Table 1, where the analytical evidence for the structure assignment is summarized. They were selected according to common structural features of the secretion components.

Isoamylalcohol (**1**), isoamylaldehyde (**2**), and isoamylacetate (**3**), the chemically most simple constituents of the investigated rove beetle glands, occurred as minor components mainly in the secretion of *Creophilus maxillosus*. Besides these compounds and the alcohol **7** all other acyclic, short-chain volatiles of the Staphylinina secretions were ketones and for the most part methyl-branched alkanones (Figure 3): 4-methyl-3-hexanone (**4**), identified by Fish and Pattenden (1975), and the homologous 4-methyl-3-heptanone (**5**) were shown to be the main volatiles responsible for the typical, sweetish odor of the species belonging to the *Ocypus*-subgenus *Goerius*. In contrast to these alkanones, which are characterized by a McLafferty ion at *m/z* 86, compound **6** showed a corresponding rearrangement fragment at *m/z* 72. The low intensity of this signal and the short retention time indicated that substance **6** was 5-methyl-3-hexanone and not the analogous linear 3-heptanone. This branched

ketone was the only alkanone in the Staphylinina secretions that was accompanied by detectable amounts of its corresponding alcohol, 5-methyl-3-hexanol (**7**). The secretion components **8** and **9**, sharing a McLafferty rearrangement fragment at *m/z* 58, were shown to be 2-heptanone (**8**) and 6-methyl-2-heptanone (**9**), which had already been found in the *Ontholestes murinus* abdominal gland by Dettner and Schwinger (1986). A weak signal at *m/z* 58 and the molecular ion M^+126 indicated, that compound **10** was an unsaturated ketone with the formula $C_8H_{14}O$ and a carbonyl function at the C-2-atom. 6-Methyl-5-hepten-2-one, which would have been a very plausible secretion component of the iridoid-producing Staphylinina glands, in analogy to the data of dolichoderine ants (Blum and Hermann, 1978), showed a nearly identical mass spectrum but had a longer retention time as the alkenone **10**. Because of the absence of further authentic methylheptenons, no concrete structure can be shown for this substance.

Other types of volatiles present in the abdominal gland secretions of the investigated Staphylinina species were cyclic and spirocyclic compounds: The gland constituents **11** and **12** had a molecular ion at *m/z* 82 and a mass spectrum with very few intense fragments in common. Although neither substance could be identified definitively for lack of the authentic compounds, the different intensity of the M^+ion (15% in component **11** vs. 90% in substance **12**) and the different base peak (*m/z* 67 vs. *m/z* 53) indicated that component **11** was a methylcyclopentene and compound **12** a methylfuran. The spirocycles identified in the Staphylinina secretions had two different ring systems (Figure 3) but showed a common pair of diagnostic fragments at *m/z* 112 and 115. (*E, E*)-2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane (**13**) and the trimethyl-1,7-dioxaspiro[5.5]-undecane **15** had already been described for the *Ontholestes murinus* secretion (Dettner and Schwinger, 1986). Compound **14** of *Ontholestes tesselatus* might be a diastereomer of the spiroacetal **13**, but this hypothetical structure assignment could not yet be confirmed. In contrast to the dioxaspiroundecanes **13–15**, the newly found spirocycles of the *Staphylinus caesareus* secretion showed a typical, intense fragment at *m/z* 155 (M^+-29). While the substances **16** and **17** could be identified as (*E,E*)- and (*E,Z*)-2-ethyl-7-methyl-1,6-dioxaspiro[4.5]decane, the spiroacetal **18** might well have a quite different ring structure, which still needs to be elucidated.

Apart from the methylcyclopentanoid monoterpenes, which will be described separately, the Staphylinina secretions contained mainly well-known natural products such as α-pinene (**19**), neral (**21**), geranial (**22**), nerol (**24**), or citronellol (**25**), and one monoterpenoid ester, (*E*)-8-oxocitronellylacetate (**26**), (Figure 3) which is closely related to 8-oxocitronellal, a probable precursor of the iridoids (Cavill and Clark, 1971). Although present only as minor com-

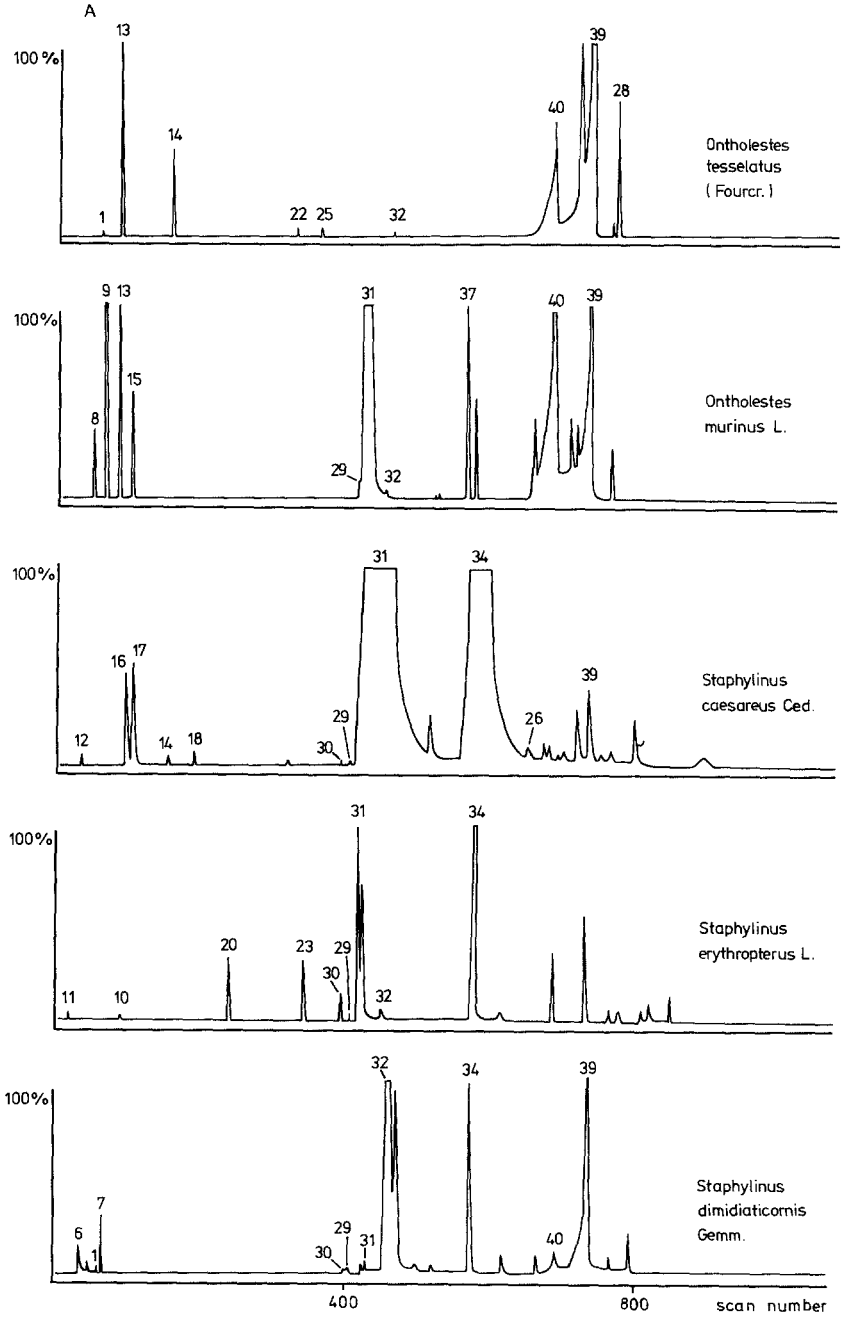


FIG. 2. Total ion monitor chromatograms (for column conditions see Methods and Materials) obtained by solid injeciton of the secretion fluid of 10 Staphylinina species of the genera *Ontholestes*, *Staphylinus*, *Ocypus*, *Creophilus*, and *Parabemus*; the numbers refer to Tables 1 and 2.

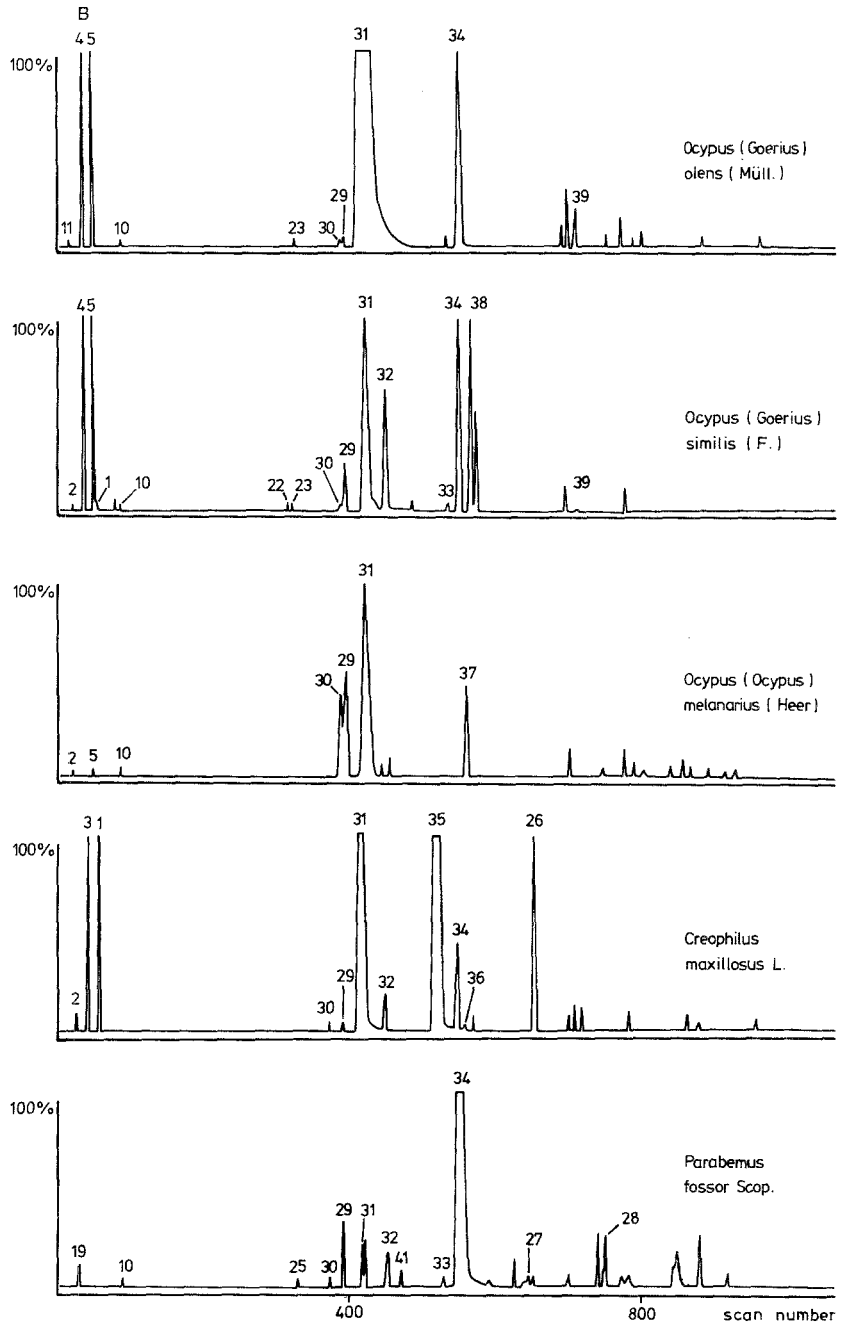


FIG. 2. Continued.

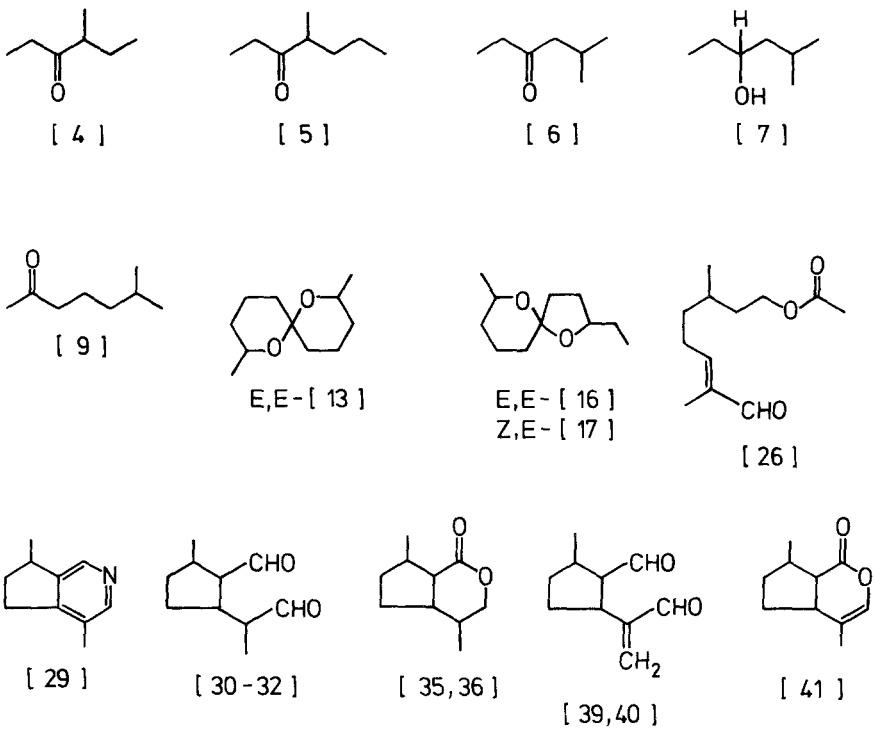


FIG. 3. Representative defensive components of the Staphylinina abdominal glands (the numbers refer to Table 1).

pound of *Parabemus fossor*, α-pinene (**19**) is quite remarkable, because it is responsible for the very peculiar, penetrating odor of the abdominal gland secretion.

A series of substances that are probably derived from fatty acid metabolism was found in the Staphylinina secretions, but for only two of these compounds could a structure proposal be made. Although the injection of the authentic ethyl esters of palmitoleic acid and oleic acid gave identical mass spectra and retention times, the concrete position of the double bond of the unsaturated esters **27** and **28** cannot be determined by GC-MS. Therefore these secretion components are listed in Table 1 as ethylhexadecenoate (**27**) and ethyloctadecenoate (**28**).

The majority of the principal components of the Staphylinina secretions belonged to the iridoid group and was characterized by an intense signal at

m/z 81, indicating the methylcyclopentanoid structure. With the exception of nepetalactone (**41**), where an authentic sample was available, all identified structures (Figure 3) were determined by coinjection with defensive secretion mixtures containing already known iridoid components. Furthermore, most iridoids occurred in two or three diastereoisomeric forms, recognizable by the clearly different retention times of substance peaks with very similar mass spectra. For lack of stereoselective syntheses that could have provided authentic diastereomers, these secretion components are listed in Table 1 with one name and marked by the suffix I, II, and III without giving any concrete stereochemical information. Actinidine (**29**), the only nitrogen-containing aromatic component of the Staphylinina secretions, and iridodial, which was detected in three stereoisomeric forms (**30–32**), were identified with the aid of earlier publications on the defense chemistry of *Ocypus olens* (Abou-Donia et., 1971), *Creophilus maxillosus* (Jefson et al., 1983), and *Ontholestes murinus* (Dettner and Schwinger, 1986). While the alkaloid **29** was present in the analyzed secretions only in very small amounts, one of the ubiquitous iridodials (for the most part substance **31**) played an important role as major component in nine of the 13 investigated secretion mixtures. In all these cases iridodial was accompanied by at least one further main compound that eluted after the saturated dialdehyde. Very often this part was taken over by the monoterpene **34**, which showed the typical fragment at *m/z* 81 with an intensity of 80%, and therefore was included into the iridoid group, although no concrete structural information could be gained from the mass spectrum. Being connected by a very similar fragmentation pattern with the obvious ion series *m/z* 139 (M^+-29), 124, and 109, the terpene **34** and the trace component **33** were regarded as stereoisomeric forms of one substance. Dihydronepetalactone, which had already been described as main secretion substance of *Creophilus maxillosus* (Jefson et al., 1983), also was found in two stereoisomeric forms (**35, 36**). Moreover one of them (**35**) was shown to be the iridoid replacing iridodial as major component in the secretion mixtures of the members of the *Ocypus* subgenus *Pseudocypus*. Although the iridoids **37** and **38** shared the ion series *m/z* 67, 81, 95, and 109 and the molecular weight of 168, only substance **38** showed a general fragmentation pattern that agreed very well with the mass spectral data reported for iridomyrmecine (Stenhagen et al., 1974). Nevertheless the great similarity of the mass spectra of stereoisomeric iridoids does not yet allow identification of component **38** definitively as iridomyrmecine without comparison of the retention times of substance **38** and the authentic iridoid lactone. In contrast, in the case of the dolichodials **39** and **40**, a clear structure assignment was possible, because the ventral gland secretions of some Tenthredinidae larvae, from which this unsaturated iridoid dialdehyde had already been reported (Boevé et al., 1984), were

TABLE 1. CHEMICAL COMPOSITION OF ABDOMINAL GLANDS OF STAPHYLININA[a]

Number	Compound	Scan (sec)	Evidence				Mw (%) M^+	Mass spectral data m/z (%)
			EI	CI	t_r	p		
1	Isoamylalcohol	58	+	+	+	+	88(0)	71(5), 70(35, M-18), 69(5), 57(12), 56(7), 55(100), 54(3), 53(4), 51(3), 50(3)
2	Isoamylaldehyde	27	+	+	+	+	86(0)	87(2, M + 1), 71(31, M-15), 69(17), 58(100), 57(36), 56(4), 55(8), 53(10), 51(8), 50(10)
3	Isomylacetate	42	+	+	+	+	130(0)	131(1, M + 1), 115(1, M − 15), 87(11), 71(8), 70(68), 69(11), 61(26), 58(4), 56(6), 55(100), 53(6)
4	4-Methyl-3-hexanone	37	+	+	+	+	114(3)	115(8), 86(13), 85(3), 58(4), 57(100), 56(9), 55(6), 53(3), 51(1), 50(1)
5	4-Methyl-3-heptanone	46	+	+	+		128(9)	129(16), 86(40), 85(14), 71(33), 70(12), 69(8), 57(100), 56(17), 55(26), 53(8)
6	5-Methyl-3-hexanone	36	+	+	+		114(6)	99(3), 86(1), 85(19), 72(6), 58(4), 57(100), 56(2), 55(2), 53(1), 50(1)
7	5-Methyl-3-hexanol	66	+	+	+		116(0)	98(1, M − 18), 87(10), 71(3), 70(7), 69(100), 59(81), 58(11), 57(23), 56(5), 55(9), 53(3)
8	2-Heptanone	52	+	+	+	+	114(0)	115(3, M + 1), 99(5, M − 15), 85(6), 72(10), 71(23), 59(19), 58(100), 55(10), 53(5), 52(5), 51(5)
9	6-Methyl-2-heptanone	65	+	+	+	+	128(0)	113(2, M − 15), 110(7), 95(17), 85(9), 71(17), 70(12), 69(8), 59(12), 58(100), 57(7), 55(12)
10	Methylheptenone	83	+	+			126(6)	111(11), 108(8), 83(35), 81(8), 71(66), 69(11), 67(27), 56(7), 55(100), 53(11)
11	Methylcyclopentene	23	+	+			82(15)	81(8), 69(7), 68(4), 67(100), 66(4), 65(15), 54(5), 53(7), 51(6), 50(6)
12	Methylfuran	26	+				82(90)	83(5), 81(55), 79(3), 63(1), 55(5), 54(18), 53(100), 52(11), 51(32), 50(37)

13	(*E*,*E*)-2,8-Dimethyl-1,7-dioxaspiro[5,5]-undecane	85	+	+	+	+	184(3)	115(97), 114(59), 112(100), 97(55), 84(25), 83(31), 71(17), 70(17), 69(78), 55(65)
14	Unknown spiroundecane	156	+	+			184(0)	115(88), 114(39), 112(37), 97(61), 84(9), 83(11), 73(22), 71(15), 69(100), 55(59)
15	2,2,8-Trimethyl-1,7-dioxaspiro[5,5]-undecane	105	+	+		+	198(0)	183(11, M − 15), 129(63), 128(35), 115(23), 112(66), 111(27), 84(66), 83(100), 82(23), 69(68), 55(98)
16	(*E*,*E*)-2-Ethyl-7-methyl-1,6-dioxaspiro[4,5]-decane	87	+	+			184(0)	185(15), 155(34), 115(85), 112(58), 97(67), 85(50), 83(39), 69(100), 57(48), 55(92)
17	(*Z*,*E*)-2-Ethyl-7-methyl-1,6-dioxaspiro[4,5]-decane	98	+	+			184(0)	185(9), 155(30), 115(84), 114(32), 112(56), 97(54), 85(51), 83(30), 69(100), 57(37), 55(69)
18	Unknown spiroacetal	181	+	+			184(0)	155(19), 115(87), 114(32), 112(43), 97(62), 95(21), 85(44), 83(29), 69(100), 57(35), 55(85)
19	α-Pinene	30	+	+	+		136(4)	121(9), 93(100), 92(48), 91(71), 79(44), 77(42), 67(16), 65(13), 53(15), 51(18)
20	Unknown terpene	218	+	+			138(24)	123(52, M − 15), 109(13), 95(67), 81(19), 79(18), 77(15), 67(100), 55(24), 53(27), 51(17)
21	Neral	295	+	+	+		152(0)	123(3, M − 29), 109(26), 94(36), 83(26), 82(23), 81(24), 69(100), 67(52), 59(25), 53(37), 51(23)
22	Geranial	307	+	+	+		152(2)	137(9), 123(6), 109(11), 94(16), 84(21), 83(17), 81(14), 69(100), 67(29), 55(13), 53(26), 51(19)
23	Unknown terpenoid alcohol	314	+	+			154(0)	136(11, M − 18), 126(30), 111(100), 93(49), 91(34), 81(65), 79(60), 69(42), 67(57), 55(90), 53(34)
24	Nerol	353	+	+	+		154(0)	136(6, M − 18), 121(19), 93(33), 92(10), 91(10), 80(15), 77(9), 69(100), 68(39), 67(39), 53(20)
25	Citronellol	363	+	+	+		156(0)	138(5, M − 18), 95(31), 82(28), 81(59), 71(22), 69(69), 68(20), 67(100), 56(20), 55(64), 53(20)
26	(*E*)-8-Oxocitronellylacetate	653	+	+	+	+	212(0)	152(15, M − 60), 97(51), 95(90), 82(37), 81(70), 79(37), 69(36), 67(100), 55(87), 53(40)

TABLE 1. Continued.

Number	Compound	Scan (sec)	Evidence				Mw (%) M^+	Mass spectral data m/z (%)
			EI	CI	t_r	p		
27	Ethylhexadecenoate	671	+	+			282(0)	237(7, M − 45), 236(9), 194(8), 152(13), 96(35), 95(32), 84(31), 83(37), 81(41), 70(28), 69(39), 67(53), 55(100)
28	Ethyloctadecenoate	773	+	+			310(0)	265(7, M − 45), 264(6), 222(7), 125(11), 98(22), 96(36), 84(29), 83(37), 81(32), 70(24), 69(60), 67(43), 57(23), 55(100)
29	Actinidine	383	+	+		+	147(55)	146(27), 132(100), 131(23), 130(14), 117(82), 77(20), 65(15), 63(12), 51(29), 50(16)
30	Iridodial (I)	372	+	+			168(0)	135(18), 81(69), 79(46), 71(38), 69(29), 67(100), 58(66), 57(40), 55(63), 53(40)
31	Iridodial (II)	397	+	+		+	168(5)	135(29), 81(94), 79(51), 71(49), 69(42), 67(100), 58(66), 57(47), 55(90), 53(47)
32	Iridodial (III)	429	+	+			168(0)	135(15), 95(28), 93(31), 81(100), 79(35), 67(67), 58(40), 57(32), 55(64), 53(34)
33	Unknown monoterpene (I)	542	+	+			168(0)	135(1), 109(17), 83(16), 81(26), 79(23), 68(16), 67(100), 57(69), 55(84), 54(20), 53(18)
34	Unknown monoterpene (II)	550	+	+			168(3)	151(11), 139(46), 109(88), 95(66), 81(80), 79(48), 69(46), 67(68), 55(100), 53(48)
35	Dihydronepetalactone (I)	510	+	+		+	168(0)	169(21), 113(49), 95(70), 82(23), 81(100), 79(22), 69(33), 67(84), 55(28), 53(27)
36	Dihydronepetalactone (II)	576	+	+			168(0)	169(2), 113(42), 110(25), 95(58), 81(100), 79(22), 69(30), 68(20), 67(73), 55(27), 53(24)
37	Unknown iridoid	566	+	+			168(0)	139(4, M − 29), 109(18), 95(30), 82(13), 81(67), 79(17), 69(17), 68(25), 67(100), 55(31), 53(22), 51(10)

38	Iridomyrmecine-like iridoid	580	+	+			168(0)	109(33), 95(67), 82(14), 81(43), 79(14), 69(17), 68(22), 67(100), 55(27), 53(18)
39	Dolichodial (I)	736	+	+		+	166(0)	148(5, M − 18), 109(31), 95(24), 83(39), 82(24), 81(29), 79(31), 67(43), 55(100), 53(44), 51(23)
40	Dolichodial (II)	684	+	+			166(0)	151(3, M − 15), 148(4), 109(24), 95(47), 91(31), 83(49), 82(33), 81(26), 79(34), 67(61), 55(100), 53(56), 51(35)
41	Nepetalactone	448	+	+	+		166(44)	123(55), 109(36), 95(75), 94(36), 81(84), 80(59), 79(39), 69(75), 67(100), 55(48), 53(63)

[a] The analytical evidence for structure assignment is summarized; the numbers refer to GC, Figure 2. Evidence: EI/CI—structure was identified only by the mass spectral data, t_r—retention time and mass spectra of the sample agreed with available authentic compound; p—secretions with definitively identified compound (references cited in the text) were used for direct comparison.

available for comparative injection. It is of interest that diolichodial was the only major iridoid in the *Ontholestes tesselatus* secretion and occurred together with a second main compound, iridodial, in the *Ontholestes murinus* gland, but was present only in lesser amounts or even lacking in the secretion mixtures of the remaining species, which had iridodial and the monoterpene **34** as major iridoids.

DISCUSSION

Because of their unprotected abdomens, almost all rove beetle subfamilies have independently evolved abdominal defensive glands that differ not only in their site and morphology but also with regard to their secretion chemistry: While some of these defense systems are based upon quinones (Aleocharinae, Oxytelinae) or iridoids (Staphylininae, Xantholininae) as major compounds, other staphylinid taxa use predominantly acids and alkenals (Omaliinae, Proteininae) or highly specialized substances such as the piperidine derivative stenusine (Steninae) in their secretions (Dettner, 1987).

In the course of the present investigation of the chemical defense of 13 Staphylinina species, more than 41 components with a great structural diversity were found to be distributed very heterogeneously within the analyzed secretion mixtures (Table 2). The major components of these secretions are iridoids, and especially iridodial is used within this Staphylininae subtribe to a much greater extent than in all other iridoid-producing rove beetle taxa. The finding that iridodial is the connecting link of all secretions investigated agrees very well with the biogenetic scheme proposed for iridoids by Cavill and Clark (1971), where this saturated iridoid dialdehyde has a key position, too. Besides iridoids, some ketones such as the *Ontholestes murinus* substance 6-methyl-2-heptanone are derived from acyclic monoterpenes in this biosynthetic concept, and this idea makes it possible to trace back the majority of the Staphylinina defense chemicals to a common monoterpene pool formed via the so called "mevalonate pathway."

Nevertheless, the fact that many intermediates of one biogenetic chain are found together in the abdominal gland reservoirs is not the only explanation for the observed diversity of the secretion substances and the heterogeneity of their distribution within the investigated rove beetle subtribe. Another possibility is that the strong tendency of the major species components, iridoidal and dolichodial, towards polymerization could have led to a primary fixative function of these dialdehydes, as proposed by Pavan and Trave (1958). Then the evolution of a variety of structurally different active substances would have been a

logical consequence of the need to improve the defensive potential of the secretion mixtures. As a result each component of the iridoid-dominated secretions would have at least a weak biological activity, as is found in the Staphylinina secretions, while the minor compounds of other defense systems (for example the quinone system) are mere solvents acting only indirectly as wetting agents, fixatives, or penetration-promoting fluids. Although this hypothesis could be correct in the case of the Staphylinina, it must be tested carefully by bioassays, because the complexity of a defensive secretion mixture must not depend on the presence of a fixative component, and until now too few toxicity studies on iridoids have been performed to decide whether iridodial and dolichodial have a defensive function exceeding the mere "carrier role."

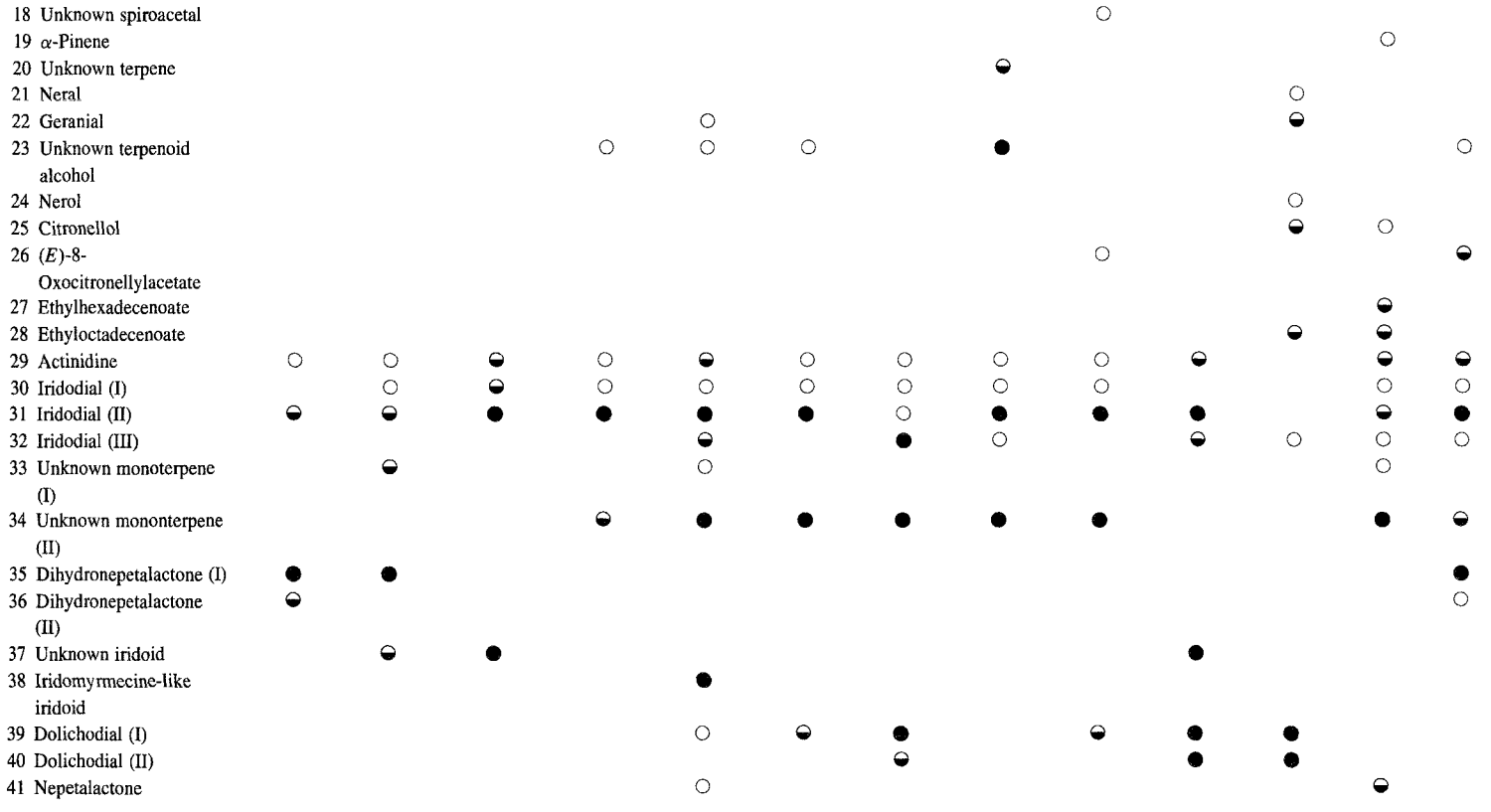
The fact that species of one Staphylinina genus use quite different active substances in combination with the common iridoids leads to specific chemical patterns, which even seem to have some chemotaxonomic value. Nevertheless this consideration needs to be confirmed by detailed study of the abdominal gland morphology in combination with phylogenetic data concerning other characters which are still in progress.

A probable biological activity of many Staphylinina alkanones is the knockdown activity (Cavill and Clark, 1971), but also a direct toxic effect is possible as was demostrated by Saslavasky and Ishay (1973) for 5-methyl-3-hexanone, an exocrine product identified for the first time in the *Staphylinus dimidiaticornis* secretion. Apart from their defensive activities against predators, alkanones can have an intraspecific function, too. 2-Heptanone is a well-known pheromone of many Hymenoptera, 4-methyl-3-hexanone, present in secretions of some Formicidae, Mutillidae, and Phalangiidae (Blum, 1981), was demonstrated to be an alarm pheromone of *Atta* ants, which have even developed chiral receptors for this substance (Riley et al., 1974). 4-Methyl-3-hexanone, found together with the homologous methylheptanone in the secretions of two *Ocypus* species and of some Formicidae (Blum, 1981) could have a similiar activity that has not yet been tested.

While little is known about small cyclic compounds of insect secretions, the rove beetle spiroundecanes described for the first time by Dettner and Schwinger (1986) already have been reported as pheromones of some Andrenidae, Anthophoridae (Wheeler and Duffield, 1988), Ichneumonidae (Davies and Madden, 1985), and of females of two *Dacus* species (Baker and Bacon, 1985). The newly identified spirodecanes of *Staphylinus caesareus* occur in the mandibular glands of Andrenidae and Ichneumonidae and also have been identified in the venom glands of some Vespidae (Wheeler and Duffield, 1988). The function of these compounds in the rove beetle abdominal glands, which have a primary defensive role, still needs to be elucidated.

TABLE 2. CHEMICAL PATTERNS OF DEFENSIVE SECRETIONS OF 13 STAPHYLININA ROVE BEETLES[a]

	Ocypus (Pseudocypus) *aenaeocephalus* (Deg.)	*Ocypus* (Pseudocypus) *fuscatus* (Grav.)	*Ocypus* (Ocypus) *melanarius* (Heer)	*Ocypus* (Goerius) *ophthalmicus* Scop.	*Ocypus* (Goerius) *similis* (F.)	*Ocypus* (Goerius) *olens* (Müll.)	*Staphylinus dimidiaticornis* Gemm.	*Staphylinus erythropterus* L.	*Staphylinus caesareus* Ced.	*Ontholestes murinus* (L.)	*Ontholestes tesselatus* (Fourcr.)	*Parabemus fossor* Scop.	*Creophilus maxillosus* L.
1 Isoamylalcohol					○		○				○		◒
2 Isoamylaldehyde			○		○								○
3 Isoamylacetate													◒
4 4-Methyl-3-hexanone					◒	◒							
5 4-Methyl-3-heptanone			○		●	●							
6 5-Methyl-3-hexanone							◒						
7 5-Methyl-3-hexanol							◒						
8 2-Heptanone									○	○			
9 6-Methyl-2-heptanone										◒			
10 Methylheptenone			◒		○	○		◒				○	
11 Methylcyclopentene					○	○		◒					
12 Methylfuran									○				
13 (*E*,*E*)-2,8-Dimethyl-1,7-dioxaspiro[5,5]-undecane									○	●	◒		
14 Unknown spiroundecane									○		◒		
15 2,2,8,-Trimethyl-1,7-dioxaspiro[5,5]-undecane										○			
16 (*E*,*E*)-2-Ethyl-7-methyl-1,6-dioxaspiro[4,5]-decane									◒				
17 (*Z*,*E*)-2-Ethyl-7-methyl-1,6-dioxaspiro[4,5]-decane									◒				



[a] The relative amounts of the components are indicated by ●: main compound, ◒: minor compound, ○: trace compound; they were evaluated by comparison of all analyses performed in the course of this investigation and therefore sometimes do not agree totally with the situation shown in Figure 2 for single injections).

One of the various biological activities of monoterpenes, which are very old natural products well known from many plant and animal sources, is the repellency against ants reported for α-pinene by Honda (1983) in the course of the study of the osmeteria of some *Papilio* larvae. This bicyclic gland constituent of *Parabemus fossor* also was isolated from the poison gland of *Myrmicaria natalensis* (Formicidae), from the frontal glands of termitide soldiers (Blum, 1981), and from secretions of some plant bugs (Rhopalidae) (Aldrich et al., 1979).

Two acetates, detectable as minor components only in the abdominal gland secretion *Creophilus maxillosus*, have been shown to be effective repellents for ants (Jefson et al., 1983): their activity was comparable to (*E*)-2-hexenal and salicylaldehyde [in the case of (*E*)-8-oxocitronellylacetate] or even *m*-cresol (in the case of isoamylacetate). This defense potential is probably the reason for the occurrence of isoamylacetate in the defensive secretion of some Hemiptera (Blum, 1981), while the honeybee uses this substance as an alarm pheromone that controls aggression behavior (Avitabile, 1973).

Apart from its occurrence in the abdominal glands of the Staphylininae and Xantholininae (Dettner, 1983), iridodial is known within the Coleoptera only from single species of the genera *Deleaster* (Staphylinidae) (Dettner et al., 1985), *Dischirius* (Carabidae) (Moore and Brown, 1979), and *Aromia* (Cerambycidae) (Vidari et al., 1973). Furthermore, this dialdehyde is a widespread secretion compound of dolichoderine ants (Blum and Hermann, 1978), where an isomer variation within three different genera was described (McGurk et al., 1968), comparable to our data for the *Staphylinus* species. Apart from its fixative properties, iridodial shows good repellency against ants (Jefson et al., 1983). The corresponding unsaturated iridoid dialdehyde of the Staphylinina secretion bouquet, dolichodial, that also had been reported from secretions of the phasmid *Anisomorpha buprestoides* (Meinwald et al., 1962), some Tenthredinidae larvae (Boevé et al., 1984), and the anal glands of formicid ants (Blum, 1981) might have a comparable defensive activity that has not yet been tested. Actinidine was found only in the secretions of staphylinids belonging to the subfamilies Staphylininae and Xantholininae and was supposed to be the main toxic component of the Philonthina rove beetles (Dettner, 1983). While many iridoid lactones are found in plants such as *Nepeta cataria* or *Actinidia polygama*, these substances are distributed quite erratically within insects: Dihydronepetalactone seems to be restricted to the defensive secretion of some Staphylinina species [genera *Creophilus* and *Ocypus (Pseudocypus)*] and was shown to exhibit a very good repellency against ants by Jefson et al. (1983). Nepetalactone, naturally occurring in catmint plants, is assumed to be a broad-spectrum insect repellent, but became known mainly because of its bizarre effect

on the behavior of cats (Eisner, 1964). Apart from its occurrence in some Staphylinina glands, this unsaturated iridoid lactone was detected only in the secretions of the *Graeffea crouani* (Blum, 1981) and *Megoura viciae* (Aphidae) (Dawson et al., 1987) up to now. The saturated lactone, iridomyrmecine, shows an insecticidal activity exceeding even the efficiency of DDT, but this extraordinary toxicity is very dependent on the stereoisomerism: for isoiridomyrmecine only an antibiotic effect is demonstrable (Pavan, 1975). This finding clearly shows the importance of an exact structure determination of iridoids such as Staphylinina substance **38**. Moreover, it emphasizes the need for stereospecifically synthesized authentic iridoids as references for analyses and as test substances for bioassays, which could lead to an improved understanding of iridoid-dominated defense systems such as the abdominal glands of the Staphylinina rove beetles.

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COMPARISON OF TECHNIQUES FOR EXTRACTING VOLATILE COMPOUNDS FROM CONIFER NEEDLES

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Abstract—Five extraction techniques were used to obtain quantitative measurements of monoterpenes from blue spruce and grand fir needles. Multivariate analysis of variance indicated that there was a significant difference in the amount of each monoterpene extracted by using different techniques. For both species, an 8-hr steam distillation of 10 g of material yielded the greatest amount of each monoterpene. This technique was also the most reproducible in that the coefficients of variation were lowest. A rapid steam distillation was relatively effective for grand fir, but not for blue spruce. A CO_2 extraction was unreliable for both species.

Key Words—Monoterpenes, *Abies grandis*, *Picea pungens*, steam distillation, extraction, secondary chemicals, grand fir, blue spruce, coefficient of variation, MANOVA.

INTRODUCTION

The analysis of terpenoid products of plant origin has been used in many studies and for several decades. Earliest use of these chemicals can be attributed to

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Baker and Smith (1920), who attempted to distinguish species of *Eucalyptus* based on the volatile constituents and characteristic odor. Since that time, advances in analytical techniques, particularly gas chromatography, have led to the widespread use of terpenoids for studies ranging from chemosystematic investigations to ecological studies. Once genetic control of terpenes had been established (Hanover, 1966, 1971; Squillace, 1971; Hiltunen et al., 1975), studies of conifer terpenes proceeded at a rapid rate to elucidate chemotypes or explain differences based on geographic origin (Wilkinson et al., 1971; Lester, 1974, von Rudloff, 1975; Schafer and Hanover, 1986).

More recently, these chemicals have been associated with adaptive significance, particularly regarding their role in pest and pathogen resistance and insect deterrence or attraction (Bridges, 1987; Ishikawa et al., 1987; Harborne, 1989). Studies of monoterpenes in relation to mechanisms involving plant–herbivore interactions have been avidly pursued in recent years (e.g., Mattson et al., 1988; Bernays, 1989). Effect of plant monoterpenes on ruminant feeding and relationships between insect herbivory and terpene level are among the aspects studied in ecological investigations of terpenoids (Elliot and London, 1987; Mihaliak et al., 1987).

Many studies have used the cortical or xylem oleoresin, or exudate, of conifers for terpene analysis; others have used extracts from different tissues. Squillace (1976) indicated that foliage terpenes may be more reliable than other sources for quantifying and describing terpene and genetic relationships. Foliage terpenes have been used widely for chemosystematic studies (von Rudloff, 1975, 1976; von Rudloff et al., 1988) and investigations related to tree improvement (Kim et al., 1976; Rehfeldt and von Rudloff, 1978). Terpenes in particular and secondary chemicals in general are widely used in species characterization and identification (Bongarten and Hanover, 1982).

In studies using foliar terpenes, there has been little uniformity of method of extraction from the needles. A wide variety of techniques ranging from simple solvent extractions to more elaborate steam distillations have been used to collect monoterpenes (Schonwitz et al., 1987). Extraction technique represents only one way in which error is introduced in terpene analysis. Errors in interpretation, statistical methods, and chromatography have been the topic of recent research (Birks and Kanowski, 1988; Raffa and Steffeck, 1988).

Although there is ample literature comparing quantitative methods for extracting and analyzing volatiles in foods (particularly head space analysis) and in aqueous systems, less is known about methods used for direct collection of essential oils from the foliage of plants. Moore (1980) found that steam distillation of blue spruce, *Picea pungens* Engelm., foliage for 8 hr in a circular still was a more reproducible technique for collecting monoterpenes than either solvent extraction with pentane or steam distillation for longer or shorter periods of time. Since that time, several new techniques have become available, includ-

ing a solvent extraction with liquid CO_2 and microdistillation techniques such as that described by Godefroot et al. (1981), which allows collection of oil from several foliage samples in a few hours.

The study reported here compares these new techniques to 8-hr steam distillations and to solvent extraction with hexane. The techniques were compared for reproducibility and for selectivity of specific monoterpenes. An attempt also was made to modify the procedures to use foliage samples of 10 g or less. Two species were used in these experiments: grand fir, *Abies grandis* (Dougl.) Lindl. and blue spruce, *Picea pungens*.

METHODS AND MATERIALS

Plant Material

Foliage was collected from five blue spruce trees growing at the Tree Research Center of Michigan State University, East Lansing, Michigan. The foliage was transported to the laboratory in Dry Ice and stored in a freezer (−11°C) until it was extracted and analyzed. The foliage was thoroughly mixed for homogeneity, and subsamples were removed to determine moisture content and total yield of oil. Grand fir foliage was collected from five trees at the Kellogg Experimental Forest, Augusta, Michigan, and was handled in the same manner as the blue spruce foliage. For both species, foliage was collected at midcrown in late summer from current year's growth.

Distillations

Eight-Hour Steam Distillation. Distillations were performed in circular steam-distillation units. Five distillations were carried out with 2 g and five with 10 g of foliage. The distillate was collected in 4 ml hexane. Menthone was added to the samples as an internal standard, and the samples were stored at −11°C until they were analyzed.

Solvent Extraction with Hexane. Five 2-g samples of foliage were crushed coarsely with a mortar and pestle in liquid nitrogen. The samples were still cold when extracted with 10 ml of hexane containing the internal standard. The foliage remained in the solvent for 30 min; the hexane was removed with a pipet and stored at −11°C until analyzed. Preliminary analysis revealed that a 30-min incubation was comparable to the quantity extracted with 24-hr incubation.

Rapid Steam Distillation. Five 2-g samples were crushed in liquid nitrogen as above. Each sample was transferred to a 100-ml two-necked round-bottom flask while still frozen, and 1 ml of hexane was added to the flask. This flask was clamped above a heating mantle at low heat and connected to a downward condenser. The bottom half of a 10-ml Mixxor extraction apparatus was placed

at the end of the condenser to collect the condensate. Steam was introduced through the second neck of the round bottom flask until 9 ml of condensate had been collected. The internal standard then was added to the condensate, and the aqueous portion of the condensate then was extracted with the hexane portion using the Mixxor. The hexane was collected and stored at −11°C until analyzed.

Liquid CO_2 Extraction. Extractions with liquid CO_2 were performed in a high-pressure Soxhlet extraction apparatus supplied by J&W Scientific (Folsom, California 85630).[6] Five 2-g samples of needles were ground in liquid nitrogen as before. The samples were transferred to a small Soxhlet extractor and were placed between pieces of glass wool. The extraction vessel was charged with liquid CO_2 and pressurized for 90 min. Periodic measurements indicated that the pressure inside the extractor fluctuated between 950 and 1200 psig. After the extraction, the unit was cooled in an ice bath and then slowly vented. A solid ball of CO_2, which contained the extracted terpenes, formed inside the round-bottom flask. The extract was dissolved in 4.0 ml of hexane as the CO_2 sublimed. When all the CO_2 had sublimed, the internal standard was added, and the samples were stored at −11°C until analyzed.

Analysis of Essential Oils

All analyses were conducted by using capillary gas chromatography with a flame ionization detector (FID) and a 0.21-mm-ID fused-silica column of 50% Carbowax and 50% methyl silicone (J&W Scientific, DX-3). Helium was used as the carrier gas with the following temperature program: 70°C for 8 min and then increased to 220°C at 6°C/min. Integrator data were compiled, and the amount of each terpene was calculated on a dry-weight basis. The monoterpenes were identified using a combination of GC-IR, GC-MS, and comparison of retention times with known standards.

Statistical Analysis

Multivariate analysis of variance (MANOVA) was used to test the terpene variables as a group to see if techniques, species, or their interactions differed significantly (Gill, 1978). A significant main effect or interaction would indicate that as a group the terpene variables were different for various levels of the significant factor. If an effect was found to be significant, multiple univariate analyses of variance were conducted with individual terpene variables used as

[6]The use of trade, firm, or corporation names is for the information and convenience of the reader. Such use does not constitute an official endorsement by the U.S. Department of Agriculture of any product or service to the exclusion of others that may be suitable.

the dependent variable and the same effects as before (technique, species, and interaction). The means for significant univariate effects were separated by using the protected least-significant-difference test (LSD). The coefficient of variation was used as an index of reproducibility and precision of each technique.

RESULTS AND DISCUSSION

The quantity of essential oils in each of these species differs widely. On the average, grand fir yielded 10 times the monoterpenes yielded by blue spruce (Figures 1 and 2). Visually, several points are apparent. The rapid steam technique was of little value in extracting monoterpenes from spruce needles, whereas for grand fir foliage, this technique was comparable to the solvent extraction. Each type of distillation yielded considerably more essential oils for grand fir than for spruce, but the variation in yield among spruce extractions is much greater. For grand fir, the 8-hr steam distillations, both 2 and 10 g, produced the greatest amount of all monoterpenes; and there was minimal difference in absolute value between the two methods. For the spruce foliage, the 10-g 8-hr steam distillation extracted the greatest amount of each monoterpene, followed closely by the solvent extraction and the CO_2 technique.

Multivariate analysis of variance (MANOVA) indicated significant differences for each species and for each monoterpene among technique means (Wilk's lambda, $P < 0.0001$). In other words, the different techniques do not

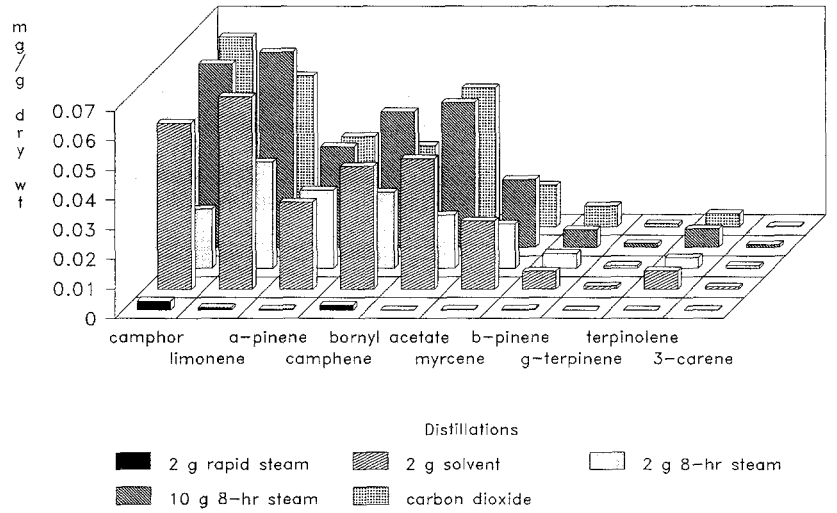


FIG. 1. Terpenes extracted from spruce needles with each of five techniques.

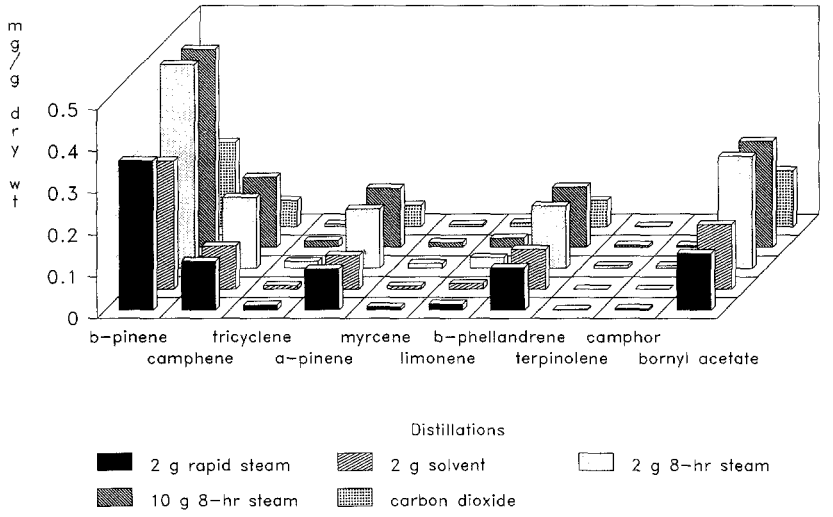


FIG. 2. Terpenes extracted from grand fir needles with each of five techniques.

extract a comparable amount of a given monoterpene. Since the MANOVA had significant effects, a univariate analysis of variance was conducted for each terpene variable to determine which techniques produced the differences. As in the MANOVA, the effects in the univariate analysis of variance were technique, species, and their interaction. For both species every monoterpene showed significant difference among the five techniques.

For spruce, the rapid steam distillation typically extracted significantly less than the other techniques, which were comparable in extraction efficiency. However, there were a couple of exceptions. For yield of 3-carene, the rapid steam, solvent, and CO_2 extractions were not different from each other, but were significantly lower than the other two techniques. For camphor, rapid steam and 2-g 8-hr distillations showed no difference in yield between them, although they yielded significantly less than the other techniques.

The analysis for grand fir monoterpenes was more complex. The CO_2 technique appeared to be less reliable and extracted lower quantities of most monoterpenes than the other techniques. There was no significant difference between the 2-g 8-hr distillation, the 10-g 8-hr distillation, the solvent extraction, and the rapid steam distillation for tricyclene, α-pinene, camphene, β-pinene, myrcene and terpinolene. That is not to say that these techniques are all comparable and are reliable means of extracting these terpenes, especially considering the reproducibility.

The coefficient of variation (CV) is a measure of the reproducibility of a measurement and can be used to compare techniques. Wide variation appears

to be pervasive throughout studies of terpenoid compounds (Hall and Langenheim, 1986; von Rudloff et al., 1988). Coefficients of variation ranging from 1% to well over 100% have been reported. Variation can also result from seed source and species differences.

Blue spruce extractions produced relatively high (>30%) CVs, except for the 8-hr steam distillation (Table 1). Although the 2-g distillation produced less than the 10-g distillation, the two techniques are equally reproducible, except for 3-carene and myrcene. By virtue of the lower CV, myrcene appears to be reproducibly extracted with the 2-g distillation, whereas 3-carene is more reproducibly extracted using 10 g of foliage.

For grand fir, only the CO_2 technique resulted in high variation. The 10-g 8-hr distillation was the only technique producing low CV values for grand fir. Although the CVs for the solvent extractions were all less than 20%, this technique does not extract terpinolene or camphor (Table 2). Generally, the CVs cannot be used to draw strong conclusions since these are based on only five observations, but are useful in conjunction with the other statistical techniques.

There is little support for use of solvent extraction since not all monoterpenes are recovered. However, if a grand fir study concerns only select monoterpene(s) other than terpinolene and camphor, the solvent method may be an economical and adequate extraction to use. Solvent extraction may have advantages over steam distillations where water acidity in the latter may cause a reduction in differential diffusion of terpenes (Koedam, 1982). Moreover, the

TABLE 1. AVERAGE VALUE AND COEFFICIENT OF VARIATION OF MONOTERPENES OF BLUE SPRUCE FOLIAGE EXTRACTED WITH VARIOUS TECHNIQUES

	Rapid steam	Solvent	8-hr (2 g)	8-hr (10 g)	CO_2
	mg/g[a] (%CV)	mg/g (%CV)	mg/g (%CV)	mg/g (%CV)	mg/g (%CV)
Camphor	0.0033a (51.1)	0.0563bc (52.0)	0.0201ab (9.1)	0.0620c (14.0)	0.636bc (83.4)
Limonene	0.0008a (47.8)	0.0651bc (34.3)	0.0364b (5.6)	0.0664c (8.5)	0.0505bc (60.1)
α-Pinene	0.0004a (100)	0.0293b (32.7)	0.0264b (7.3)	0.0342b (8.3)	0.0305b (58.6)
Camphene	0.0017a (33.4)	0.0415b (34.0)	0.0256b (7.1)	0.0456b (6.8)	0.0275b (85.9)
Bornyl acetate		0.0443a (39.7)	0.0178b (6.8)	0.0489a (6.8)	0.0468a (75.1)
Myrcene	0.0002a (86.5)	0.0229b (28.8)	0.0150b (1.4)	0.0232b (11.9)	0.0143b (80.6)
β-Pinene	0.0003a (51.9)	0.0057b (37.7)	0.0045b (6.0)	0.0063b (6.0)	0.0074b (59.2)
γ-terpinene		0.0010a (67.1)	0.0009a (16.4)	0.0013a (9.0)	0.0011a (59.1)
Terpinolene	0.0000a (100)	0.0062b (30.8)	0.0035b (4.9)	0.0063b (9.0)	0.0044b (81.8)
3-Carene	0.000a (100)	0.0008abc (98.2)	0.0010bc (25.9)	0.0013b (8.0)	0.0004ac (100)

[a]Values are mean dry weight. Values, within a row, followed by the same letter are not significantly different ($P > 0.05$).

TABLE 2. AVERAGE VALUE AND COEFFICIENT OF VARIATION OF MONOTERPENES OF GRAND FIR FOLIAGE EXTRACTED WITH VARIOUS TECHNIQUES

	Rapid steam	Solvent	8-hr (2 g)	8-hr (10 g)	CO_2
	mg/g[a] (%CV)	mg/g (%CV)	mg/g (%CV)	mg/g (%CV)	mg/g (%CV)
β-Pinene	0.3576ab (17.8)	0.3074ab (14.1)	0.4880a (36.9)	0.4742a (8.8)	0.2008b (69.7)
Camphene	0.1167ab (20.3)	0.1035ab (16.0)	0.1689a (39.6)	0.1684a (9.9)	0.0625b (66.2)
Tricyclene	0.0116ab (19.4)	0.0089ab (20.7)	0.0163a (38.8)	0.0164a (10.3)	0.0058b (75.0)
α-Pinene	0.0984ab (18.9)	0.0810ab (16.5)	0.1422a (38.0)	0.1416a (9.5)	0.0511b (71.2)
Myrcene	0.0081ab (18.2)	0.0076ab (14.1)	0.0116a (36.1)	0.0115a (10.6)	0.0048b (63.3)
Limonene	0.0147abc (16.2)	0.0136bc (16.2)	0.0258a (37.0)	0.0233ab (11.8)	0.0095c (59.3)
β-Phellandrene	0.1018ab (16.9)	0.0953b (12.7)	0.1490a (34.9)	0.1446a (8.6)	0.0628b (62.7)
Terpinolene	0.0017ab (57.8)		0.0068a (32.8)	0.0057a (8.2)	0.0014b (72.4)
Camphor	0.0041a (23.9)		0.0063b (28.2)	0.0046b (14.6)	0.0037a (42.3)
Bornyl acetate	0.1358ab (18.6)	0.1386c (15.5)	0.2684a (24.9)	0.2540ab (11.0)	0.1344b (61.3)

[a] Values are mean dry weight. Values, within a row, followed by the same letter are not significantly different ($P > 0.05$).

results of Schonwitz et al. (1987) suggest that rearrangements of terpenes do not occur during extraction with cold pentane, comparable to extraction with cold hexane.

Analysis of the array of monoterpenes of a particular species is the intent of many chemosystematic studies. For grand fir, the 10-g 8-hr distillation is clearly superior for this purpose, followed by rapid steam distillation. The 8-hr distillations are the most suitable for blue spruce.

Clearly, the structure of needles must affect the extractions of monoterpenes. Several hours are required for blue spruce extractions, perhaps because of the build-up of epicuticular waxes on this species. The monoterpenes of grand fir needles, with fewer waxes, may be sufficiently extracted with a 5-min rapid steam distillation or 30-min solvent extraction. The most reliable data, however, will be obtained with 8-hr distillations. The results presented here are valid for the two genera investigated, and they illustrate how different species respond to various extraction procedures. Researchers studying monoterpenes should closely assess the technique appropriate for the given species as well as direct the analysis toward the questions addressed. Such differences must be considered in any study of foliar terpenes.

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CHEMICAL ECOLOGY OF CANARIAN LAUREL FOREST: TOXIC DITERPENES FROM *Persea indica* (LAURACEAE)[1]

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Abstract—The tree species *P. indica* (Lauraceae) is an important endemism in the Canary Islands laurel forest and can readily be distinguished by its defoliated appearance due to the seasonal action of wild rats (*Rattus rattus*), which eat the plant and become intoxicated. These observations and the phytochemical interest of this plant species led us to study the potentially toxic chemicals responsible for such action. We found that an ethanolic extract of *P. indica* and its water fraction were toxic when injected into laboratory mice. The mice also died after ingestion of the stems and showed a significant preference for those extracted and rehydrated with an 8% aqueous extract solution when compared with the water control. Two compounds that have been isolated from the toxic fraction and identified by spectroscopic methods are the polyhydroxy pentacyclic diterpenes ryanodol and cinnceylanol. Possible ecological implications are discussed.

Key Words—*Persea indica*, Lauraceae, ryanodol, cinnceylanol, X-ray analysis, *Rattus rattus*, toxicity, mice feeding trials.

INTRODUCTION

We began the study of the chemical ecology of the Canarian laurel forest because of its importance as a relict flora and the potential presence in this plant species

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of unknown chemical products with ecological and economic relevance. Moreover, plant species are being lost at a much faster rate than rates of evolutionary replacement (Wilson, 1988), and loss of a species means loss of chemicals potentially unique in nature (Eisner, 1989).

The flora of Macaronesia (Canary Islands, Madeira, and Azores) is an ancient remnant of the Tertiary flora, which has been preserved to the present day and affords us a graphic example of what flora of the Tethyan region was like in the first half of the Tertiary period. Towards the end of this period, the subtropical forest flora was displaced because of changes in the climatic zones. The probable southern migration of a cooler, drier climate led to the development of the modern Mediterranean sclerophyllous vegetation (see Bramwell, 1976).

For this project, the tree species *Persea indica* was chosen for two reasons: (1) Its present day distribution represents only a part of its former range, so it can be considered a good example of paleoendemism (Bramwell, 1972), which has never been studied from a phytochemical point of view. (2) This tree has a characteristic defoliated appearance resulting from the seasonal action (April, May) of a wild forest rat (*Rattus rattus*) that obtains cortical tissue by selecting terminal twigs from larger branches and biting through the stem at an average distance of 20 cm from the tip of the branch. The rough outer bark is then removed to expose the phloem, cambium, and some xylem. It also has been observed that these wild rates become intoxicated from eating the plant, occasionally falling from the trees.

In the study of plants and animals that feed on them, the notion that secondary chemicals play an important role in food selection by vertebrate herbivores has become widely accepted (Harborne, 1982). These products have been used to explain herbivore preferences at many levels: colubus monkeys that avoid the phenolic barrier present in the abundant tree species of their environment by eating low-tannin seeds and leaves of relatively rare deciduous trees (McKey and Gartland, 1981): squirrels that select low-monoterpene ponderosa pines (Farentinos et al., 1981); browsing mammals that choose palatable shrubs based on leaf structure and phenolic compounds (Simonetti et al., 1984; Cooper and Owen-Smith, 1985); Canada geese avoiding herbs with high concentrations of tannins (Buschsbaum and Valiela, 1987); elephants avoiding species with high total phenols, steroidal saponins, and lignin (Jachmann, 1989); or desert wood rats that avoid feeding on creosotebush (*Larrea tridentata*) phenolic resin to the point of energy imbalance (Meyer and Karasov, 1989).

We selected this plant–herbivore system to demonstrate that *P. indica* contained secondary chemicals that could interfere with its selection as a food source by rodents having negative effects on them and to isolate and identify the active products of this plant due to its potential phytochemical importance.

METHODS AND MATERIALS

Plant Material and Extraction. P. indica branches were collected at El Moquinal, Monte de Las Mercedes, Tererife, in October 1988. Air-dried, chopped, terminal twigs ($\geq$20 cm, 627 g) were extracted with EtOH for six days in a Soxhlet apparatus. The cold extract was filtered and then concentrated in vacuo to give a syrup gum.

Fractionation of Extract. The recovered EtOH extract (29 g, yield 4.6%) was chromatographed on a silica gel column and eluted with a light petroleum–EtOAc–MeOH gradient. A total of 16 fractions were eluted (light petroleum–EtOAc. 85:15 to 0:100; EtOAc–MeOH, 95:5 to 50:50) and bioassayed for mice toxicity. Part of the toxic fraction (eluted with light petroleum–EtOAc, 55:45) gave a white precipitate (A) and a yellow supernatant (B). By dissolving A in aqueous MeOH, allowing the solvent to evaporate slowly at room temperature, and repeating the process, pure compound I was obtained.

Fraction B (1.2 g) was analyzed by droplet countercurrent chromatography (DCCC) in a model A apparatus (Tokyo Rikakikai Co., Japan). The sample was dissolved in a mixture of both mobile and stationary phase and injected into the apparatus using a 20-ml sample chamber. The flow rate was 8–10 ml/hr, and the eluates were collected in 3 to 6-ml fractions.

The fractions were monitored by TLC on silica-gel sheets (Schleicher-Schull). The solvent system was $CHCl_3$–MeOH–H_2O, 35:65:40 and the compounds were detected with a mixture of H_2O–AcOH–H_2SO_4, 80:15:5 (oleum reagent).

Thirteen fractions were obtained and the active ones (6–9) combined and rechromatographed by DCCC (625 mg). Pure crystals of compound II were obtained from recrystallization on MeOH of the active fractions collected.

Physical Data. Melting points were taken on a Kofler hotplate apparatus and are uncorrected. [^{1}H] NMR spectra were recorded on a Bruker P 200 SY spectrometer, py-d_5 and CD_3OD were used as solvents. Mass spectra were taken in a Hewlett-Packard 5995 and high-resolution mass spectra (HRMS) in a VG Micromass Zab-2F spectrometer.

Ryanodol (I). m.p. 345–347°C (from methanol–water) [lit. synthetic I, 266–267°C (Wiesner et al., 1967), 267–268°C (Belanger et al., 1979)], [^{1}H] NMR (CD_3OD, 200 MHz) δ: 3.80 (1H, d, J = 10 Hz, 10-H), 3.74 (1H, s, 3-H), 1.34 (3H, s, 17-H), 1.05 (3H, d, J = 6 Hz, 21-H), 1.00 (3H, d, J = 6 Hz, 19-H), 0.95 (3H, d, J = 6 Hz, 18-H), 0.89 (3H, s, 20-H); [^{1}H] NMR (py-d_5, 200 MHz) 4.57 (1H, d, J = 7 Hz, 10-H), 4.26 (1H, s, 3-H), 2.15 (3H, s, 17-H), 1.50 (3H, d, J = 6 Hz, 21-H), 1.28 (6H, d, J = 6 Hz, 18-H and 19-H), 1.14 (3H, s, 20-H). MS, *m/z* (rel. int.): 400 (M^+) (7), 382 (2), 357 (16), 339 (100), 321 (63), 303 (49), 293 (36), 261 (25), 257 (14), 247 (18), 243

(20), 229 (11), 195 (10), 165 (9), 155 (12). HRMS, calcd. for $C_{20}H_{32}O_8$ 400.2097, found 400.2090.

Cinnceylanol (II). m.p. 140–141°C (from methanol) [lit. m.p. 139–142°C (Yagi et al., 1980)]; [^{1}H] NMR (CD_3OD, 200 MHz) δ: 3.80 (1H, d, J = 10 Hz, 10-H), 2.40, and 1.76 (each 1H, d, J = 15 Hz, 3-H), 1.31 (3H, s, 17-H), 1.00 (6H, d, J = 6 Hz, 18-H and 19-H), 0.95 (3H, d, J = 6 Hz, 21-H), 0.86 (3H, s, 20-H). MS, *m/z* (rel. int.) 384 (M^+) (2), 366 (5), 349 (5), 348 (17), 341 (14), 331 (16), 330 (69), 323 (100), 315 (17), 305 (24), 289 (75), 287 (20), 271 (22), 259 (20), 223 (13), 217 (11), 195 (15). HRMS, calcd. for $C_{20}H_{32}O_7$ 384.2166, found 384.2157.

X-Ray Analysis of Ryanodol (I). The molecular formula was $C_{20}H_{32}O_8$ and the molecular weight 400.468 g/mol. Accurate cell parameters of the unit cell [a = 16.283 (1) Å, b = 13.065 (1) Å, c = 9.112 (1) Å, V = 1936.6 (3) Å^3] were determined on a crystal measuring 0.30 × 0.26 × 0.20 mm, by least-square analysis of setting angles of 35 reflections ($10 < \Theta < 36$) using graphite monochromated CuK_α radiation (μ = 8354 cm^{-1}), automatically located and centered on a Philips PW-1100 diffractometer. The space group was $P2_12_12_1$, and z = 4. Two reflections were measured every hour to ascertain crystal stability, and no significant variation was observed, with $\omega/2\Theta$ scan and scan width 1.50. The intensities of 3534 reflections were collected and 3449 reflections were considered in accordance with $I \geq 2\sigma(I)$. They were subsequently corrected for Lorentz and polarization effects but no absorption corrections were applied.

The structure was solved by direct methods (MULTAN 80), using the solution with the highest figure of merit from 36 different sets (2200 relationships, E_{min} = 1.5). Successive difference Fourier revealed the positions of all non-H atoms. The positional and thermal parameters were refined using full matrix least-squares. After several difference Fourier maps, which allowed the localization of all H atoms, several anisotropic least-squares cycles were carried out, but the H atoms were considered as fixed isotropic contributors. Function minimized $\Sigma w(|F0| - |Fc|)^2$, where w was empirically calculated, not to give trends in $\langle w\Delta^2F \rangle$ vs. $\langle F0 \rangle$ and vs. $\langle \sin \Theta/\lambda \rangle$. Convergence was reached at R = 3.6% and R_w = 3.8%.

The absolute configuration was determined by comparing the 40 (ΔFc 0.052) more relevant Bijvoet pairs, giving the following average Bijvoet differences: 0.156 for the right enantiomer vs. 0.395 for the wrong one.

Toxicity Bioassay. This bioassay was design to detect toxic compounds in the different fractions obtained during the purification and partitioning processes. Laboratory mice (18–22 g) were injected intraperitoneally with either the EtOH extract (10 mg), the silica-column fractions (10 mg), or the droplet countercurrent chromatography (DCCC) fractions (1 mg) with 1% Tween 60

(Merck) or physiological saline solution (9/00 ClNa) as the carrier vehicle, compared with appropriate controls, and their mortality recorded at 24 hr.

The reported LD_{50}s are based on plots of dose vs. probit percent mortality calculated according to Tallarida and Murray's procedure (1986). Three to five mice, caged together, were used for each concentration of product assayed, five concentrations of compound I were tested (13.37, 17.54, 18.78, 20.01, and 20.50 mg/kg) and six of compound II (3.61, 5.82, 8.39, 12.12, 26.83, and 35.58 mg/Kg).

Feeding Experiments. All the feeding experiments were run at room temperature and at a 12 : 12 light–dark photo-period.

The survivorship of mice feeding ad libitum on *P. indica* dried stems was calculated by comparing their performance with that of mice feeding ad libitum on mice chow for six days. One set of three mice for the control, and two more sets for the treatment were used after a 24-hr starvation period. Each set was kept in a 240 × 135 × 130-cm-high cage.

A second feeding experiment was designed to check for food preferences. Extracted *P. indica* twigs were oven dried (60°C, 48 hr) and rehydrated with increasing concentrations of an aqueous extract obtained by dissolving the EtOH residue in distilled water (10% w/v). The concentrations used were: 4, 6, and 8% w/v, and the results compared with 100% water-rehydrated twigs as control. Petri dishes containing weighed amounts o the differently treated stems were offered individually to the mice for 30 min. Three mice per cage and five cages per treatment were used in the three feeding trials. The cages were as previously described, and food consumption was calculated gravimetrically.

RESULTS AND DISCUSSION

From the field observations we suspected that *P. indica* was toxic to rodents. To check for this activity, we injected laboratory mice intraperitoneally with a crude ethanolic extract and its water fraction, observing that the injected mice suffered muscular convulsions prior to death. To check if *P. indica* stem tissue also was toxic to mice after ingestion, they were fed ad libitum on dried stems. The animals started showing similar symptoms to those observed after intraperitoneal injection of the toxic extracts after three days, reaching a 100% mortality rate on the sixth day of treatment (Figure 1). On the other hand, it is known that when animals encounter toxic foods, they will initially eat small quantities of it and then refuse to feed (Meyer and Karasov, 1989).

To verify that the toxic fraction also was causing a feeding response, we designed the trials described in Methods and Materials. We found that mice ate significantly more ($P \leq 0.005$) of the 6% and 8% aqueous extract rehydrated

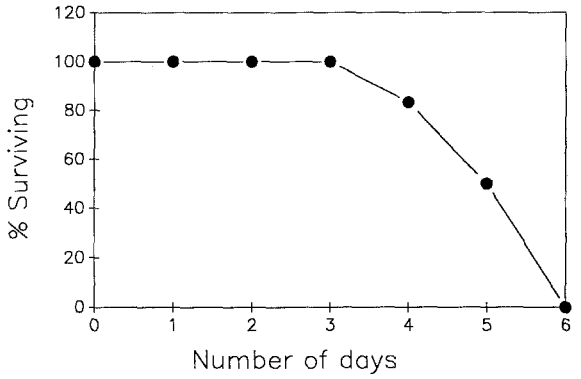


FIG. 1. Survivorship curve for laboratory mice fed on *P. indica* dried stems, expressed as percentage of control. $N = 9$ animals (3 for control and 6 for treatment).

stems than of the water controls, and they did not shown any preference below the 4% aqueous extract treatment when compared to the control (Figure 2).

Besides the toxicity that the stems had on laboratory mice, preliminary field observations on the seasonal feeding preferences of the wild rats on this

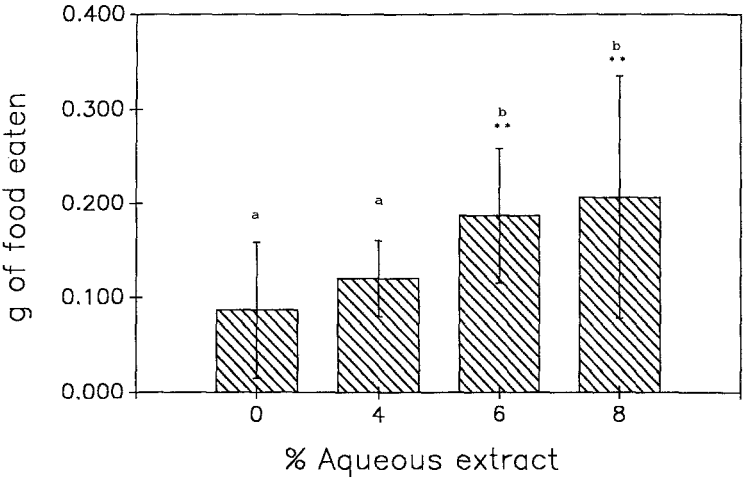


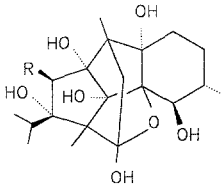
FIG. 2. Amount of *P. indica* stems eaten after rehydration with increasing concentrations of its aqueous extract. Treatments were as follows: 0, control with water and rehydration; 4, 4% aqueous extract; 6, 6% aqueous extract; 8, 8% aqueous extract ($N = 15$ animals per treatment). $^{*}P < 0.05$, $^{**}P < 0.05$, t test significance level when different from the control. $^{a,b}P < 0.05$ significance level for between-treatment comparisons.

tree and our laboratory data suggest that there are components in *P. indica* stems that induce a positive feeding response in rodents.

These preferences can be governed by a balance between beneficial nutritional components and toxic secondary chemicals. The aqueous extracts used in the food-choice experiment contained toxic products along with sugars, proteins, and minerals that could explain the response observed for this extract. There are many examples of herbivores' food choices governed by the ratio between beneficial nutritional components and toxic secondary chemicals: howler monkeys influenced by the protein–fiber ratio and secondary chemicals of their food (Milton, 1979), bullfinches preferentially feeding on seeds with a high fat–phenolic compounds ratio (Greig-Smith, 1988), etc. Allelochemicals are also likely to cause depletion of several mineral nutrients, causing herbivores to develop an appetite for minerals (Freeland et al., 1985).

Carbon–nutrient balance of the plants determined by resource availability in the environment must play an important role in determining levels and types of plant defense (Bryant et al., 1985; Bryant, 1987), and hence herbivore impact (Niemelä and Danell, 1988). Moreover, temporal variations in plant chemistry cause seasonal changes in the use of different species of plants by herbivores (Buchsbaum and Valiela, 1987: Cooper et al., 1988). This type of change could explain the seasonal feeding behavior of *Rattus rattus* on *P. indica* that we have observed in the field. Quantitative analysis of the seasonal variations of this plant's toxic compounds and beneficial elements (nitrogen, water, etc.) is needed to test this hypothesis.

In order to isolate the toxic products involved in the interaction described here, we fractionated and purified the ethanolic crude extract. This procedure yielded two pure diterpenes. The less polar one was ryanodol (I, Figure 3), which was identified on the basis of the following: High-resolution mass spectrometry is in accordance with the formula $C_{20}H_{32}O_8$ and its [^{1}H] NMR spectrum is in accordance to that described in the chemical literature for one of its esters, ryanodine, if the geminal proton to the esterified alcohol and the signals of the ester group are excluded (Waterhouse et al., 1985). We decided to submit



I R = OH

II R = H

FIG. 3. Structures of ryanodol (I) and cinnceylanol (II).

this compound to X-ray analysis because only the *p*-bromobenzyl ether of ryanodol has been subjected previously to this technique (Srivastaba and Przybylska, 1968), and the previous results obtained are not useful to study the interactions between this substrate and the enzymes involved in its metabolism. The crystallographic conformation of ryanodol (I) is illustrated in Figure 4, which also shows the atom numbering and the absolute stereochemistry.

Ryanodol (I) has been previously obtained synthetically (Wiesner et al., 1967; Wiesner, 1968; Belanger et al., 1979), and is isolated now for the first time from a natural source. This diterpene was known in nature in the form of its α-pyrrole carboxylate ester derivative known as ryanodine (Wiesner et al., 1967). This is an insecticidal and toxic alkaloid, which was obtained from the plant species *Ryania speciosa* (Flacourtiaceae), a shrub native to Trinidad and the Amazon basin, which is used as ryania powder to control agricultural and garden pests (Worthing, 1979). It is highly toxic because of an action on the transverse tubular system in muscles by reducing the release of calcium from the sarcoplasmic reticulum in cardiac muscle cells (Sutko et al., 1985). Ryanodol, in contrast to ryanodine, has low toxicity to mice and little activity at mammalian receptors, yet they are potent knockdown agents for injected houseflies and cockroaches, suggesting a possible difference in the target sites if mammals and insects (Waterhouse et al., 1987).

The more polar diterpene obtained was identified as cinnceylanol (II, Figure 3) on the basis of its physical data. This compound has been isolated previously from the bark of *Cinnamonum zeylanicum* (Lauraceae) (Isogai et al., 1976), as an insecticidal compound causing abnormal ecdyses to *Bombix mori* larvae at dosages lower than 16 ppm in artificial diets (Isogai et al., 1977). This

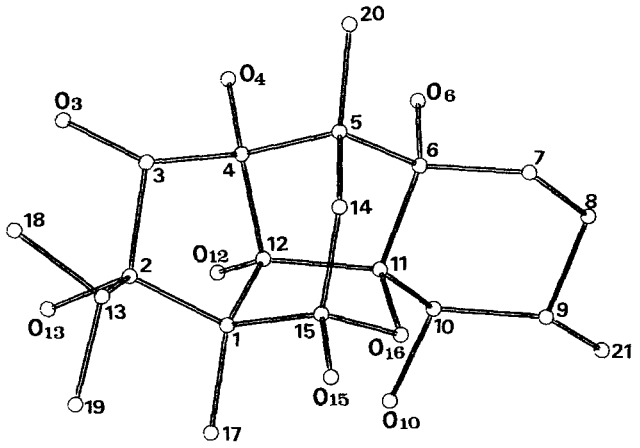


FIG. 4. Molecular structure of ryanodol (I).

TABLE 1. TOXICITY OF RYANODOL AND CINNCEYLANOL TO MICE

	LD_{50}(mg/kg)	95% limits	
		Lower	Upper
Ryanodol	17.918	16.227	19.786
Cinnceylanol	11.988	6.718	21.392

product also has been obtained from *C. cassia* as an anticomplement active component (Yagi et al., 1980). Cinnceylanol has not been described as being highly toxic to mammals, but from its structural similarities to ryanodol, it could share a similar mechanism of action.

The fact that we have found these insecticidal products in an endemic species of the island opens the possibility of exploiting their potential as natural pesticides to be used locally and emphasizes the importance of chemical screening of the biologically active products of this unique flora.

Both compounds, ryanodol and cinnceylanol, were toxic when injected intraperitoneally into laboratory mice (Table 1), showing similar symptoms to those previously described for the extracts. It is possible that *P. indica* contains toxic products other than I and II. The joint action of all these chemicals could result in a synergistic total effect since cooccuring synergists may be structurally optimized for intraspecific phytochemistry (Berenbaum, 1985). More experiments are needed to asses this possibility (i.e., comparisons of the effects of whole extracts against pure compounds at sublethal doses).

We plan to continue our search for other chemical and environmental factors governing the interaction discussed here, as well as the defensive role the above-mentioned chemicals play in the control of invertebrate herbivores.

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GLUCOSINOLATE LEVELS IN COTYLEDONS OF MUSTARD, *Brassica juncea* L. AND RAPE, *B. napus* L. DO NOT DETERMINE FEEDING RATES OF FLEA BEETLE, *Phyllotreta cruciferae* (GOEZE)

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Abstract—Sinigrin (allyl glucosinolate), the major glucosinolate in the cotyledons of *Brassica juncea* cv. Cutlass, occurred in the highest concentration and amount at seedling emergence and declined during growth. Glucobrassicin (3-indolylmethyl glucosinolate), the major glucosinolate in the cotyledons of *B. napus* cv. Westar, occurred in the lowest concentration and amount at seedling emergence. The amount of glucobrassicin per cotyledon pair increased about fourfold during 14 days of growth, but its concentration remained relatively unchanged because of "dilution" by increasing cotyledon biomass. These different glucosinolate profiles indicate a different metabolic control and different biological function for sinigrin and glucobrassicin. The flea beetle, *Phyllotreta cruciferae* Goeze, does not discriminate between cotyledons having sinigrin or glucobrassicin since the two crucifers were fed upon equally in choice tests. Restricting the concentration of sulfur in the nutrient medium accelerated the decline of sinigrin in *B. juncea* cv. Cutlass but did not alter the feeding rate of *P. cruciferae* compared to controls. Sulfur restriction reduced glucobrassicin in *B. napus* cv. Westar to undetectable levels and somewhat reduced the feeding rate of *P. cruciferae*. Nevertheless, *P. cruciferae* still fed actively on cotyledons of *B. napus* cv. Westar depleted of glucosinolates and severely damaged many of them. Since glucosinolate type and concentration had little effect on feeding response, reduction or elimination of foliar glucosinolates alone would not seem a useful strategy for protecting seedlings of these two crucifers from flea beetle damage.

Key Words—Glucosinolate, sinigrin, glucobrassicin, sulfur, crucifer, *Brassica juncea*, *B. napus*, flea beetle, *Phyllotreta cruciferae*, Coleoptera, Chrysomelidae, feeding, cotyledon.

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INTRODUCTION

Glucosinolates are thioglycosides found in all members of the *Cruciferae* examined to date including many important crops. In some crops such as mustard, mustard oils derived from glucosinolates impart a desirable pungent odor and taste, whereas in other crops such as oilseed rape, glucosinolates and their breakdown products are detrimental to the extracted meal and have now been largely eliminated by plant breeding. The significance of mustard oil glucosides as attractants and of glucosinolates as feeding stimulants for insects specializing in *Cruciferae* has been recognized from the time of Verschaeffelt (1911) and since then thoroughly documented (Thorsteinson, 1953; Wensler, 1962; Nayar and Thorsteinson, 1963; Feeny et al., 1970; Hicks, 1974; Nielsen, 1978; Dilawari and Atwal, 1987). The possible consequences of extensive reduction of the level of glucosinolates in modern cultivars on feeding by crucifer specialists and perhaps by generalists, as well, have not been studied adequately. Feeding damage caused by flea beetles of the genus *Phyllotreta* to "high" or "low" glucosinolate rape cultivars has not been found to be correlated with the glucosinolate designation (Jonasson, 1982; Larsen et al., 1985; Lamb, 1988). These conclusions, however, were based on glucosinolate levels in mature seeds or defatted seed meal, which are never eaten by flea beetles! Although the level of glucosinolates in rape seed is a valuable datum that is readily available because it is important to plant breeders and oil processors, it is now known that glucosinolates vary enormously in their occurrence, amount, concentration, and distribution within the plant (Sang et al., 1984; McGregor, 1988). Therefore, it is necessary to correlate insect feeding with the type and concentration of glucosinolates actually found in the plant tissue being consumed. In this study we document the major glucosinolates in the cotyledons of two crucifer hosts of the flea beetle *P. cruciferae* during seedling growth, when they are highly susceptible to flea beetle damage (Lamb, 1989). We further have manipulated these glucosinolate levels in the cotyledons by sulfur restriction and describe herein the effects upon feeding rates of *P. cruciferae*.

METHODS AND MATERIALS

Plants. Seedlings of mustard, *Brassica juncea* cv. Cutlass, and oilseed rape, *B. napus* cv. Westar, were grown on a modified Hoagland-type nutrient solution in which magnesium chloride replaced magnesium sulfate. Various levels of sulfur were supplied by potassium sulfate. The seedlings were grown under wide-spectrum (Gro-Lux) artificial lighting in the laboratory at 20 $\pm$ 1°C, 50–60% relative humidity and 18 hr light–6 hr dark photoperiod.

Insects. Flea beetles collected in the field from allyl isothiocyanate-baited traps (Burgess and Wiens, 1980) were maintained on radish (*Raphanus sativus*

L. cv. Round Red) in the laboratory at 20 ± 1°C (day) and 15 ± 1°C (night), 70% relative humidity, and 16:8 light–dark cycle with photoperiod from 0700 to 2300 hr. *P. cruciferae* were separated from *P. striolata* and starved for 48–72 hr (feeding test 1) or for 24 hr (feeding test 2) prior to starting the experiments. Beetles were not used more than once in any experiment.

Feeding Test 1. For experiments in which *B. juncea* cv. Cutlass and *B. napus* cv. Westar were compared in choice tests, seedlings were grown for seven days in nutrient medium with unrestricted sulfur (1.0 mM sulfate ion) in Vermiculite in 175-ml styrofoam coffee cups having a 0.5-cm-diam. hole punched in the bottom for drainage. Each cup contained two mustard seedlings and two rape seedlings in alternating order. Six cups were placed in a screened cage 30 × 30 × 30 cm and 48 adult *P. cruciferae* were introduced. After 24 hr, the beetles were removed from the plants, and the percent of the area of each cotyledon that had been eaten was estimated using a low-power dissecting microscope fitted with an optical grid. The experiment was replicated five times.

Feeding Test 2. For experiments requiring seedlings grown in nutrient media having various concentrations of sulfur (1.0, 0.1, 0.01, or 0.001 mM sulfate ion), the seedlings were grown for 10 days in glass bowls 10 cm diam. and 5 cm high filled with glass beads 0.3 cm diam.; glass beads were added to keep plants erect and to facilitate easy removal of plants without damage to roots. Nutrient solutions were removed daily by aspiration and replaced by fresh nutrient solutions. Feeding tests were conducted in circular arenas constructed from a plastic cylinder 20 cm diam. and 20 cm high fitted with fine mesh on the top. The bottom of the cylinder fitted over a rigid plastic foam base which was drilled with concentric rings of holes 1.5 cm diam. at a spacing of 2.5 cm. Seedlings with their roots intact were inserted into the holes and held in place by a split plug of soft plastic foam. The roots of the seedlings extended through the base into a pan of distilled water.

In all experiments, plants grown in the nutrient medium with unrestricted sulfur (1.0 mM) (control) were compared to those grown in one of three others having sulfate ion concentrations of 0.1, 0.01, or 0.001 mM (treatments). Three experiments, one for each treatment, were conducted for each plant species (Table 2 below). The experiments were replicated five times, each replicate consisting of an arena with four pairs of plants, one control and one treatment plant constituting a pair. Plants of a pair, matched for size as closely as possible, were positioned in adjacent holes in the arena; between pairs, however, a hole was left unused to obtain greater separation of pairs. The beetle density was set at 100/arena. All experiments were started at 1000 hr to 1100 hr and continued usually for 30 hr, but longer if plant damage at this time was insufficient for damage evaluation. During this period, the number of beetles found on individual plants were recorded three times, approximately at 5, 10, and 22 hr after the start of the experiment. At the end of the experiment the beetles were removed from the arena, and the damage to each cotyledon was estimated

visually using a rating scale from 0 (no feeding damage) to 10 (cotyledons fully eaten). The data on flea beetle numbers on plants were analyzed by paired *t* test. Since an ordinal scale was used in rating plant damage, the damage score data were subjected to nonparametric analysis using the sign test.

Extraction and Quantitation of Glucosinolates. Cotyledons (5 g) were cut from seedlings with sharp scissors and held for 5 min in 100 ml of boiling 80% (*v/v*) methanol contained in the stainless-steel cup of a Sorval homogenizer. The methanol–plant mixture then was homogenized for 1 min at maximum speed and the homogenate centrifuged at 12,000*g* to remove insoluble material. The pellet was further extracted with 100 ml of hot 80% ethanol, centrifuged as above, and the extracts combined. Methanol was removed from the combined extract on a rotary evaporator at 40°C, and the concentrated aqueous extract (ca. 30 ml) then was adjusted to contain 0.05 M barium lead acetate in a volume of 50 ml. The mixture was stirred for 1 hr at ambient temperature and then centrifuged at 12,000*g* for 10 min to remove precipitated material. Twenty milliliters of the clear supernatant were mixed with 80 ml of deionized water and applied to a column of DEAE-Sephadex A-25 (0.1 g dry wt) that had been treated with 0.5 M pyridine-acetate and equilibrated with water. The column was washed with 10 ml deionized water, 2 ml of 0.02 M pyridine-acetate and 3 ml of deionized water. Glucosinolates bound to the column were desulfated with aryl sulfatase overnight at room temperature as described by Minchinton et al. (1982) and eluted from the column by two washes of 1 ml of deionized water. Appropriate aliquots (20–100 μl) were taken for HPLC. Recoveries (92–103%) of glucosinolates as their desulfo derivatives were estimated by adding known amounts of allyl glucosinolate (to rape extracts), 3-indolylmethyl glucosinolate (to mustard extracts), or benzyl glucosinolate (to both extracts).

Desulfoglucosinolates were separated on a 250-mm LiChrospher 100 RP-18 (10 μm) column (E. Merck, Darmstadt, F.R.G.) pumped at 0.8 ml/min with a step gradient of water; 4%, 8%, and 12% acetonitrile; and monitored at 227.5 nm. An internal standard of O-nitrophenyl-β-D-galactopyranoside (Sigma Chemical Co., St. Louis, Missouri) was used in the quantitation of the desulfoglucosinolates as described by Sang and Truscott (1984) and McGregor (1985).

Allyl glucosinolate (sinigrin) was purchased from Sigma and benzyl glucosinolate was a gift from Dr. E. Underhill. The 3-indolylmethyl glucosinolate (glucobrassicin) was isolated from 1.8 kg of Savoy cabbage (*B. oleracea* cv. gemmifera) as described by Hanley et al. (1983).

RESULTS AND DISCUSSION

Glucosinolates in Mustard and Rape Cotyledons during Seedling Growth. The profile of glucosinolates in the cotyledons of the mustard, *B. juncea* cv.

Cutlass, and rape, *B. napus* cv. Westar, during seedling growth were completely different (Figures 1–3). Allyl glucosinolate was the major glucosinolate of *B. juncea* cv. Cutlass and other aliphatic, aromatic, and indole glucosinolates were either absent or occurred at concentrations too low to be detected by methods used in this study. Allyl glucosinolate occurred in the highest concentration per milligram of dry plant weight and highest amount per cotyledon pair at seedling emergence and declined both in concentration and amount during seedling growth (Figure 2). The decline in concentration with time appeared to be the more rapid, but this difference was more apparent than real and simply reflected the increasing biomass of the cotyledons during growth.

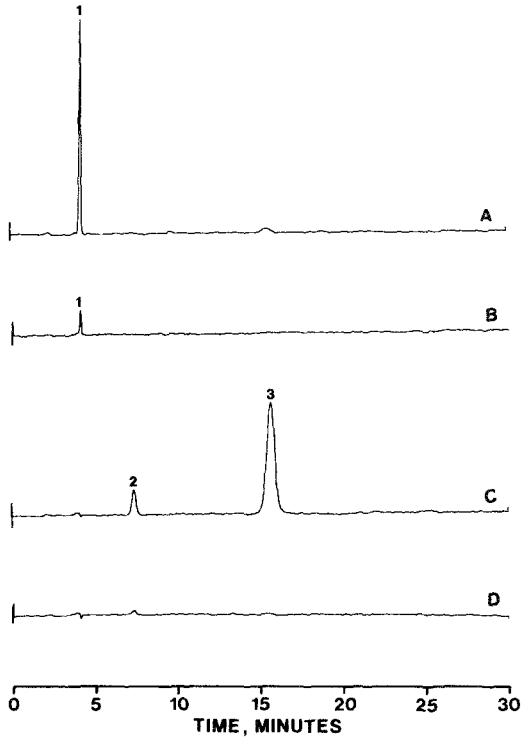


FIG. 1. HPLC profile of glucosinolates as their desulphoderivatives from the cotyledons of the mustard *Brassica juncea* cv. Cutlass (A, B) and oilseed rape, *B. napus* cv. Westar (C, D). Cotyledons were from seedlings 14 days after seeding and grown on a Hoagland-type nutrient medium having unrestricted sulfur (1.0 mM sulfate ion) (A, C) or having restricted sulfur (0.001 mM sulfate ion) (B, D). The eluted desulfoderivatives correspond to: 1, allyl glucosinolate (sinigrin); 2, unidentified; 3, 3-indolylmethyl glucosinolate (glucobrassicin). The HPLC column in A and B were eluted with water and in C and D with 12% acetonitrile. The flow rate was 0.8 ml/min in each case.

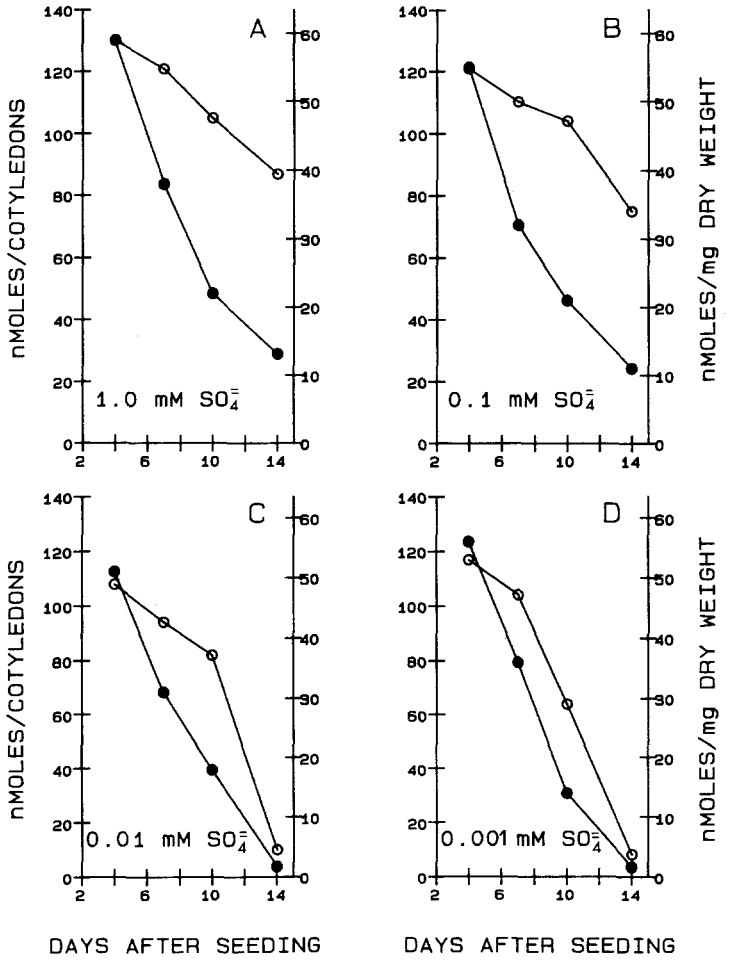


FIG. 2. Changes in the amount and concentration of allyl glucosinolate (sinigrin) in the cotyledons of the mustard, *Brassica juncea* cv. Cutlass, during seedling growth on Hoagland-type nutrient media having various concentrations of sulfate ion. Open circles, the amount of allyl glucosinolate in the cotyledons; closed circles, its concentration per unit dry weight.

The major glucosinolate in the cotyledons of *B. napus* cv. Westar was 3-indolylmethyl glucosinolate, but lesser amounts of an unidentified aliphatic glucosinolate also were seen on chromatograms (Figure 1). The cotyledons of the low glucosinolate cultivar Westar had few (or a lower concentration) of the aliphatic and aromatic glucosinolates seen in the high glucosinolate cultivar Midas (McGregor, 1988) but 3-indolylmethyl glucosinolate is a prominent

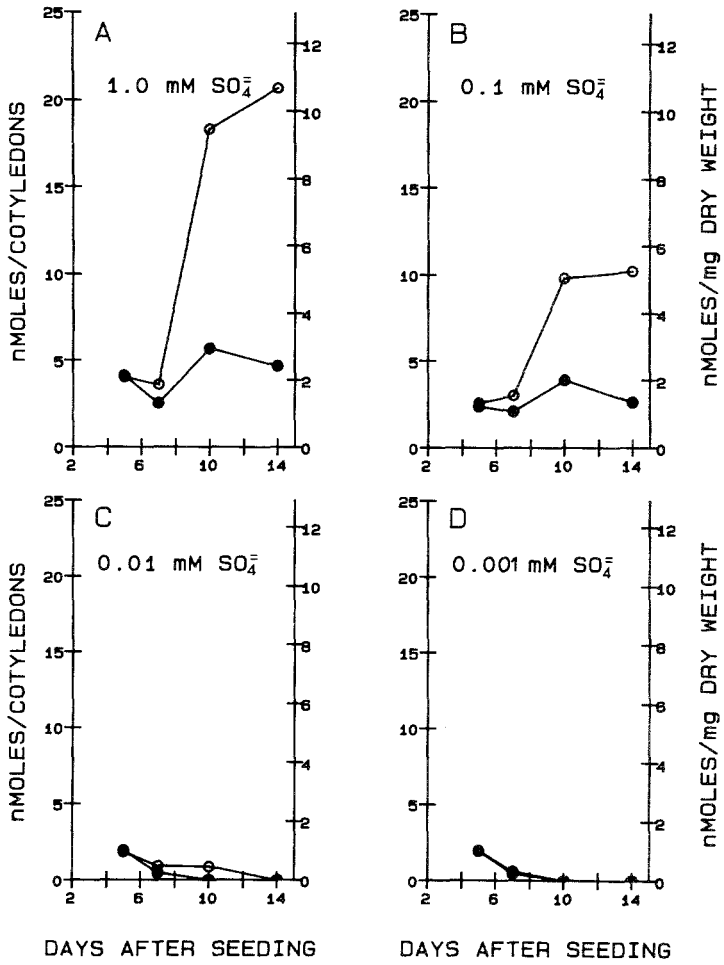


FIG. 3. Changes in the amount and concentration of 3-indolylmethyl glucosinolate (glucobrassicin) in the cotyledons of oilseed rape, *Brassica napus* cv. Westar, during seedling growth on Hoagland-type nutrient media having various concentrations of sulfate ion. Open circles, the amount of 3-indolylmethyl glucosinolate in the cotyledons; closed circles, its concentration per unit dry weight.

component of the cotyledons of the growing seedling of both rape cultivars. Unlike allyl glucosinolate in *B. juncea* cv. Cutlass, 3-indolylmethyl glucosinolate occurred in the lowest concentration per milligram of dry plant weight and lowest amount per cotyledon pair at seedling emergence. The amount of 3-indolylmethyl glucosinolate in the cotyledons of *B. napus* cv. Westar increased about fourfold during 14 days of growth but the concentration changed rela-

tively little because of "dilution" by the increasing biomass of the cotyledons (Figure 3). Similarly, when the amount of individual glucosinolates in seedlings of *B. napus* cv. Midas was expressed on a whole plant basis, aliphatic glucosinolates, 2-phenylethyl glucosinolate, and total indole glucosinolates increased during growth (McGregor, 1988).

Effect of Sulfur Restriction on Cotyledon Glucosinolate Levels. Progressive sulfur restriction reduced the level of glucosinolates in cotyledons during seedling growth but the effect was much less severe for allyl glucosinolate in *B. juncea* cv. Cutlass than for 3-indolylmethyl glucosinolate in *B. napus* cv. Westar (Figures 1–3). In the case of *B. juncea* cv. Cutlass, the decline in the amount and concentration of allyl glucosinolate in the cotyledons during seedling growth was accelerated by progressive sulfur restriction, but the effect occurred relatively slowly over a range of sulfate ion concentrations in plant nutrient solutions that differed by 1000-fold (Figure 2). By contrast, the effects of progressive sulfur restriction on the levels of 3-indolylmethyl glucosinolate in the cotyledons of *B. napus* cv. Westar during seedling growth were seen sooner and at higher sulfate ion concentrations (Figure 3). At 0.1 mM sulfate ion, the increase in the amount and concentration of 3-indolylmethyl glucosinolate was noticeably reduced compared to unrestricted sulfate ion concentration (1.0 mM) and eliminated at 0.01 mM. At sulfate ion concentrations of less than 0.01 mM, glucosinolates became undetectable in cotyledons of *B. napus* cv. Westar after 10 days of growth (Figure 3). These effects of sulfur restriction during seedling growth are consistent with de novo synthesis and accumulation of 3-indolylmethyl glucosinolate predominating in the cotyledons of *B. napus* cv. Westar and removal (by catabolism or translocation) of allyl glucosinolate predominating in the cotyledons of *B. juncea* cv. Cutlass. These different cotyledon glucosinolate profiles indicate a different metabolic control and different biological function for allyl glucosinolate and 3-indolylmethyl glucosinolate.

Sulfur restriction also reduced the dry weights of the cotyledons of *B. juncea* cv. Cutlass and *B. napus* cv. Westar, an effect that was concentration- and time-dependent (Table 1). The effect of sulfur restriction on the growing seedling, however, was relatively benign for the first 10 days after seeding, and cotyledon biomass was seldom reduced by more than 10% even in the presence of the lowest (0.001 mM) concentration of sulfate ion (Table 1). After two weeks, seedlings grown in the presence of the lowest concentrations of sulfate ion were visibly smaller, with *B. napus* cv. Westar showing the largest cotyledon weight reductions (−33%, Table 1). Classical symptoms of sulfur deficiency (purple leaf color, visibly stunted plants) did not appear until about 21 days, well after the appearance of the first true leaves (data not shown). Therefore, for feeding tests with *P. cruciferae*, we used seedlings 10 days after seeding and grown in Hoagland's nutrient medium containing different levels of sulfate ion.

TABLE 1. DRY WEIGHTS (mg) OF COTYLEDONS OF MUSTARD *Brassica juncea* CV. CUTLASS AND OILSEED RAPE *Brassica napus* CV. WESTAR GROWN ON NUTRIENT SOLUTION HAVING DIFFERENT SULFATE ION CONCENTRATIONS[a]

Plant	Days after seeding	Sulfate ion concentration (mM)			
		1.0	0.1	0.01	0.001
B. juncea cv. Cutlass	4	2.2 ± 0.3	2.2 ± 0.3 (0)	2.1 ± 0.3 (−4.5)	2.1 ± 0.4 (−4.5)
B. napus cv. Westar	5	2.1 ± 0.4	2.1 ± 0.3 (0)	2.1 ± 0.3 (0)	2.0 ± 0.4 (−4.8)
B. juncea cv. Cutlass	7	3.3 ± 0.6	3.3 ± 0.5 (0)	3.0 ± 0.4 (−9.1)	2.9 ± 0.7 (−12)
B. napus cv. Westar	7	2.8 ± 0.6	2.8 ± 0.5 (0)	2.8 ± 0.5 (0)	2.5 ± 0.6 (−11)
B. juncea cv. Cutlass	10	4.7 ± 1.0	4.7 ± 0.7 (0)	4.6 ± 0.6 (−2.1)	4.4 ± 0.9 (−6.4)
B. napus cv. Westar	10	4.8 ± 0.8	4.8 ± 1.1 (0)	4.7 ± 0.9 (−2.1)	4.4 ± 1.0 (−8.3)
B. juncea cv. Cutlass	14	6.6 ± 1.1	6.5 ± 1.2 (−1.5)	5.9 ± 1.2 (−11)	5.2 ± 1.4 (−21)
B. napus cv. Westar	14	8.5 ± 2.2	7.6 ± 1.1 (−11)	6.7 ± 1.5 (−21)	5.7 ± 1.5 (−33)

[a] Values represent the mean dry weight of the cotyledons from 74–80 plants and the standard error. Values in parenthesis represent the percent difference in dry weight compared to plants grown in the nutrient solution having 1.0 mM sulfate ion (complete Hoagland's).

Effect of Cotyledon Glucosinolates and Sulfur Restriction on Feeding Rates of P. cruciferae. Cotyledons of *B. juncea* cv. Cutlass and *B. napus* cv. Westar grown under conditions of unrestricted sulfur were fed upon equally in the two-choice test. Data for mean percent cotyledon area eaten from five replicates were: *B. napus* cv. Westar, 11.6 ± 3.3; *B. juncea* cv. Cutlass, 12.1 ± 3.6, $P > 0.05$, t test. Thus *P. cruciferae* does not discriminate between cotyledons having allyl glucosinolate or 3-indolylmethyl glucosinolate. In a similar choice test to determine host plant preference among several crucifers, Palaniswamy and Lamb (1991) found no differences between these two crucifers in the number of *P. cruciferae* and *P. striolata* found on them or in the amount of damage to the cotyledons. Our result is also consistent with artificial feeding studies that have shown that allyl glucosinolate and 3-indolylmethyl glucosinolate are both excellent feeding stimulants for *P. cruciferae* (Larsen et al., 1985). The absence of feeding discrimination between cotyledons having allyl glucosinolate or 3-indolylmethyl glucosinolate and the order of magnitude difference in their cotyledon concentrations (Figures 2 and 3) also may indicate that these substances are not primary determinants of cotyledon feeding for *P. cruciferae*.

Sulfur restriction in *B. juncea* cv. Cutlass did not alter the response of *P. cruciferae* (Table 2) in terms of the number of beetles on plants or the extent of damage to cotyledons. The number of beetles found on plants is a measure of host plant preference (Palaniswamy and Lamb, 1991). In our tests with Cutlass, beetles showed no preference to plants receiving different levels of sulfate

TABLE 2. EFFECT OF SULFATE ION CONCENTRATION IN NUTRIENT SOLUTIONS ON NUMBER OF FLEA BEETLES FOUND ON PLANTS AND AMOUNT OF DAMAGE TO COTYLEDONS OF MUSTARD, *Brassica juncea* CV. CUTLASS AND OILSEED RAPE, *B. napus* CV. WESTAR

Exp	Sulfate ion conc. (mM)	Beetles/plant[a,b] (mean)		Damage score/plant[a,c] (mean)	
		Cutlass	Westar	Cutlass	Westar
1	1.00	2.25a	2.20a	6.95a	5.90a
	0.10	1.75a	2.35a	6.85a	5.50a
2	1.00	1.70a	2.75a	5.75a	4.65a
	0.01	1.00a	2.15a	4.60a	4.30b
3	1.00	1.05a	2.40a[d]	3.50a	5.55a
	0.001	1.35a	1.40b[d]	2.13a	3.10b

[a] In each experiment, values with the same letter in the same column do not differ ($P = 0.05$).
[b] Flea beetle counts at 10 hr after beginning experiment.
[c] Damage estimate at 30 hr after beginning experiment.
[d] FB counts taken at other observational times were not significantly different.

ion. Similarly, in terms of feeding responses, beetles did not discriminate between cotyledons differing in their allyl glucosinolate concentration (Figures 1 and 2). Evidently other nutrient components of the cotyledons, such as amino acids and proteins, were not affected sufficiently to alter the feeding response of *P. cruciferae*, consistent with our observation that the degree of sulfur restriction chosen in these studies also did not much affect cotyledon biomass (Table 1). It is possible, by prolonging sulfur restriction for two or three weeks, to achieve lower concentrations of allyl glucosinolate for cotyledon feeding tests, but these conditions introduce confounding elements into the experiments, especially the progressive stunting of plant growth, and were therefore not undertaken. It is sufficient here to establish that the concentration of allyl glucosinolate in the cotyledons of *B. juncea* cv. Cutlass decreases rapidly during seedling growth and that acceleration of the natural rate of decrease by sulfur restriction did not produce cotyledons that were eaten less by *P. cruciferae*.

Sulfur restriction in *B. napus* cv. Westar to 0.01 mM did not cause any reduction in the responses of beetles. However, a further reduction to 0.001 mM significantly reduced the number of beetles on plants as well as the level of damage (Table 2). Thus, the beetles discriminated in choice tests between cotyledons that had high concentrations of 3-indolylmethyl glucosinolate and those in which glucosinolates were no longer detectable (Figures 1 and 3). Although *P. cruciferae* fed less on cotyledons depleted of glucosinolates, the beetles still consumed these cotyledons and damaged many of them severely.

Elimination of Foliar Glucosinolates as a Strategy for Controlling Crucifer Pests. In an especially convincing study of glucosinolates as flea beetle feeding stimulants, Hicks (1974) demonstrated that bean leaves, *Phaseolus vulgaris* L., normally unacceptable to adults of *P. cruciferae*, were avidly fed upon if the leaves were first made to take up allyl glucosinolate through their petioles. In this study we undertook the reciprocal experiment, the removal of glucosinolates from host plants of *P. cruciferae*, but did not achieve the reciprocal effect, i.e., the elimination of feeding on the host. According to one point of view, a plant lacking glucosinolates would not be recognized as a host plant by crucifer specialist insects (Larsen et al., 1985), a highly desirable agronomic trait. This does not appear to be the case for *B. napus* cv. Westar bred to contain low levels of aliphatic glucosinolates for commercial use and further depleted of aliphatic and indole glucosinolates in the laboratory in this study by restricting sulfur. The cotyledons of these experimental rape plants were thoroughly depleted of glucosinolates but were still fed upon, and many were still heavily damaged. Evidently, a glucosinolate feeding stimulant is not essential for *P. cruciferae* to eat the cotyledons of *B. napus* cv. Westar. It is possible to argue that although the cotyledons of *B. napus* cv. Westar were depleted of 3-indolylmethyl glucosinolate, subnanomolar concentrations may still have remained, and flea beetles may have responded to these trace concentrations in the feeding tests. Such arguments are not cogent in light of experiments by Larsen et al. (1985), showing that treatment of the cotyledons of oilseed rape cultivars with 3-indolylmethyl glucosinolate (8 mM) did not enhance their acceptability to four species of flea beetles.

A glucosinolate-derived mustard oil attractant also appears unnecessary for feeding to occur because cotyledons of *B. napus* cv. Westar that contain 3-indolylmethyl glucosinolate, which has no volatile breakdown product, are eaten as readily as the cotyledons of *B. juncea* cv. Cutlass that contain allyl glucosinolate, which breaks down to allyl isothiocyanate, a volatile and highly attractive substance to flea beetles.

P. cruciferae likely uses many chemical, visual and tactile cues in its feeding response to plants. We suspect that the cotyledons of *B. napus* cv. Westar are eaten by *P. cruciferae* because they contain no deterrent to its feeding. Our data indicate that reduction or elimination of foliar glucosinolates would not alone be a useful strategy for protecting mustard or oilseed rape from flea beetle feeding damage. It further seems unlikely that such a strategy would render large, commercial fields of these crops sufficiently less apparent (Feeny, 1976) to crucifer pests for significant reduction in feeding damage to occur. Flea beetles continue to exploit effectively the chemically modified oilseed rapes in Canada (Lamb, 1988), indicating that they have no difficulty finding or feeding upon the crop. The incorporation of feeding deterrents, antibiosis factors, or factors for increased tolerance to damage may offer a better noninsecticidal strategy for protecting these crops against insect pests.

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ALLELOPATHIC INHIBITION OF *Cynodon dactylon* (L.) PERS. AND OTHER PLANT SPECIES BY *Euphorbia prostrata* L.

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Abstract—Field observations indicated that *Euphorbia prostrata* strongly interferes with *Cynodon dactylon* (L.) Pers. Analysis of some physical and chemical soil factors indicated that competition was not the dominant factor of that interference. Soil collected from under *E. prostrata* stands was very inhibitory to seed germination and seeding growth of some of the test species including *C. dactylon*. This suggests the presence of inhibitory compounds in soil of *E. prostrata* stands. Subsequent experiments showed that aqueous extract, decaying residues, and root exudates of *E. prostrata* were inhibitory to most of the test species including *C. dactylon*. Thus, it appears that allelopathy is the major component of the interference, with competition probably accentuating its effect. It also was found that allelopathy is an important component of the interference by *E. prostrata* against *Amaranthus retroflexus*, *Medicago sativa*, and *Gossypium hirsutum*.

Key Words—Allelopathy, vegetational patterning, *Euphorbia prostrata*, *Cynodon dactylon*, *Amaranthus retroflexus*, *Medicago sativa*, *Gossypium hirsutum*, biological control.

INTRODUCTION

Allelopathy has been identified and well documented in natural and manipulated ecosystems (Muller et al., 1968; Lodhi, 1975, 1978; Gliessman, 1983; Rice,

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1984). The major impact of this phenomenon is restricted in shifting species distribution and thereby determines vegetational patterning (Rasmussen and Rice, 1971; Alsaadawi and Rice, 1982; Alsaadawi and Al-Rubeaa, 1985; Rice, 1984).

Euphorbia prostrata, which is locally called "bermudagrass cancer," is a pernicious weed in bermudagrass lawns in Iraq. The weed often expands the size of its stand from a few plants in a small area to a large area in a few months; even in a heavy stand of bermudagrass. As the *E. prostrata* spreads, it almost eliminates the bermudagrass and forms virtually pure stands. This rapid spread suggests the possibility of an allelopathic mechanism in addition to competition. Therefore, the present investigation was initiated mainly to test whether *E. prostrata* is allelopathic to bermudagrass and selected crop and weed species. Another aim was to obtain preliminary information about the possibility of using *E. prostrata* in biological control of weeds in some crops.

MATERIALS AND METHODS

Soil Property Analysis. Soils were analyzed to determine if *E. prostrata* caused marked changes in selected soil properties that could account for the change in vegetational patterning. Ten soil samples, free of litter, were collected randomly from pure stands of *E. prostrata*, and 10 similar samples were taken from adjacent stands of bermudagrass. The soils were air-dried under shade and passed through a 2-mm sieve. Soil reaction (pH), electrical conductivity (EC), and texture were analyzed by the methods outlined by Piper (1942). Concentrations of nitrate nitrogen and ammonium nitrogen were determined by the Devarda alloy method (Bremner, 1965), organic carbon by the procedure of Anderson and Harries (1967), and available phosphorus by the method of Olsen et al. (1954). Water-soluble Ca, Mg, Na, K, Fe, Cu, and Zn were determined on a Shimadzu AA 760 atomic absorption spectrometer. Chloride ion concentration was measured with an Orion pH mV meter.

Field Soil Bioassay. This experiment was conducted to exclude possible competition from *E. prostrata* and to determine if inhibitor(s) are released by *E. prostrata* into the soil and remain stable long enough to affect plant growth. Soil samples were collected from the rooting zone (0–15 cm) under *E. prostrata* and under bermudagrass stands in October 1988. The soil samples of 500 g each were placed in pots, kept under greenhouse conditions until May 1989, and then used in the bioassay. A given number of seeds of each of the test species was planted in their respective pots. Bermudagrass was used as a test species since it is associated with *E. prostrata*. *Amaranthus retroflexus* was included because it is an important weed in cultivated fields. *Gossypium hirsutum* and *Medicago sativa* were chosen as test species since they are important crops in which bermudagrass and *A. retroflexus* are often noxious weeds. The

pots were placed in a greenhouse and arranged in a complete randomized design with five replications. Seed germination was recorded after one week, and the pots were thinned to three seedlings per pot for cotton and four seedlings per pot for the other species. The seedlings were allowed to grow for an additional seven weeks, harvested, and weights of oven-dried roots and tops were determined.

Aqueous Extract Bioassay. E. prostrata plants were collected from the field, air-dried, and ground with an electrical grinder. Thirteen grams of plant powder were extracted with 210 ml of methanol for 12 hr in a Soxhlet extractor. The extract was evaporated to dryness under vacuum at 60°C and the residue was dissolved in 1300 ml of distilled water making the concentration 1%. The same amount of methanol was subjected to the same procedure and used as a control.

For the bioassay, a given number of seeds of each of the test species was planted in pots, each containing 1500 g of acid-washed quartz sand. Immediately after planting, 200 ml of the extract was added to each of the test pots. During the experiment, all pots were watered with equal amounts of distilled water alternated with equal amounts of Hoagland's solution (Hoagland and Arnon, 1950). The amounts of water and Hoagland's solution were adjusted to prevent leaching. All pots were kept in a greenhouse and arranged in a complete randomized design with eight replicates for each treatment. Seed germination was recorded after one week, and the pots were thinned to three seedlings for each species. Seedlings were allowed to grow for an additional seven weeks, harvested, separated into roots and tops and compared on the basis of oven-dry weight.

Residue Bioassay. Field measurements revealed that *E. prostrata* adds an average of 1.5 g of air-dried plant material per kilogram of soil in the top 10 cm. Therefore, to determine the effects of air-dried materials of *E. prostrata* on growth of test species at a rate of 1.5 g/kg soil, air-dried powder of *E. prostrata* plants were incorporated in a loamy soil of 1.5 g/kg soil. Air-dried peat moss was incorporated in the loamy soil at the same rate for the control. The treated and control soils were placed in pots of 1.4 kg capacity each, and a definite number of seeds of each of the test species was planted in each control and test pot. The experiment was run under greenhouse conditions using a completely randomized design with six replications. Seed germination was recorded after one week and seedlings were thinned to three per pot for cotton and four per pot for the other test species. Seedlings were allowed to grow for seven additional weeks, harvested, oven-dried, and roots and tops weighed.

Root Exudate Bioassay. Seeds of *E. prostrata* and other test species were planted in special cylindrical funnels (22 cm length × 8 cm diam.), each containing 1 kg of acid-washed quartz sand. The funnels were painted with aluminum paint to exclude light. Three days after germination, seedlings of each

species were thinned to two per pot and then the bioassay initiated. Funnels containing *E. prostrata* seedlings were fixed on a system and alternated with funnels containing seedlings of the test species. Control was accomplished by alternating funnels of each species with funnels containing acid-washed sand only. Hoagland's solution of 0.25 strength dripped from a supply reservoir placed at the top of each series into the funnels and allowed to drip from funnel to funnel by gravitational force and then into collecting reservoirs at the bottom. The solutions were transferred back to the upper reservoir two times a day. Each treatment was replicated four times. Thirty days after beginning the experiment, plants were harvested and oven-dry weight of roots, tops, and seedlings was determined.

RESULTS

Soil Property Analyses. The results revealed that there were no significant differences in soil texture, pH, EC, organic carbon, and concentration of mineral elements between *E. prostrata* stands and surrounding stands of bermudagrass (Table 1). However, concentrations of Na, C, and Mg and EC were appreciably higher in bermudagrass stands than in *E. prostrata* stands.

TABLE 1. COMPARISON OF SOIL FACTORS BETWEEN *Euphorbia prostrata* STANDS AND ADJACENT STANDS OF *Cynodon dactylon*[a]

Test factors	Values[b]	
	E. prostrata stand	*C. dactylon* stand
pH	8.09	7.84
Electrical conductivity (mS/cm)	1.62	2.9
C (%)	4.0	4.8
P (ppm)	76.0	66.0
NO-N (ppm)	48.0	50.0
NH-N (ppm)	28.0	30.8
K (ppm)	13.0	12.8
Ca (ppm)	45.9	37.0
Mg (ppm)	63.9	80.0
Na (ppm)	53.4	76.7
Fe (ppm)	0.13	0.10
Cu (ppm)	0.04	0.03
Zn (ppm)	0.10	0.08
Cl (ppm)	150.2	211.6
Sand (%)	20.0	20.5
Silt (%)	36.7	39.2
Clay (%)	43.3	40.3

[a] Means of 10 Replications.
[b] Differences are not significant at 0.05 level according to Student's *t* test.

Field Soil Bioassay. Soil collected from *E. prostrata* stands significantly reduced seed germination and growth of roots, shoots, and total weight of seedlings of bermudagrass and *A. retroflexus* (Table 2). Germination and growth of the other test species were inhibited appreciably by soil collected from *E. prostrata* stands as compared with bermudagrass stands.

Aqueous Extract Bioassay. Aqueous extract of *E. prostrata* significantly reduced germination of bermudagrass and *A. retroflexus* (Table 3), and germination of the other test species was inhibited appreciably also. The aqueous extract significantly reduced root, top, and seedling growth of bermudagrass

TABLE 2. EFFECT OF SOILS FROM *E. prostrata* STANDS (TEST) AND *Cynodon dactylon* STANDS (CONTROL) ON GERMINATION AND GROWTH OF TEST SPECIES

Test species	Treatments	Oven-dry weight (mg)[a]			Germination (% of Control)
		Root	Top	Seedling	
Cynodon dactylon	Control	16.8	9.5	26.3	100.0
	Test	5.8[b]	2.1[b]	7.9[b]	44.4
Amaranthus retroflexus	Control	15.0	25.8	40.8	100.0
	Test	3.6[b]	8.9[b]	12.5[b]	50.0
Gossypium hirsutum	Control	148.5	369.9	518.4	100.0
	Test	104.6	289.8	394.4	90.0
Medicago sativa	Control	8.3	16.9	25.2	100.0
	Test	7.1	12.9	20.0	84.0

[a] Average of five replicates.
[b] Significantly different from control at 0.05 level according to Student's *t* test.

TABLE 3. BIOLOGICAL ACTIVITY OF AQUEOUS EXTRACT OF *E. prostrata* L. AGAINST GERMINATION AND GROWTH OF TEST SPECIES

Test species	Treatments	Oven-dry weight (mg)[a]			Germination (% of Control)
		Root	Top	Seedling	
Cynodon dactylon	Control	13.9	20.9	34.8	100.0
	Test	5.2[b]	7.4[b]	12.6[b]	37.0
Amaranthus retroflexus	Control	17.5	41.8	59.3	100.0
	Test	31.6	27.8[b]	59.4	36.2
Gossypium hirsutum	Control	249.7	695.0	944.7	100.0
	Test	218.8	580.0[b]	798.8[b]	86.6
Medicago sativa	Control	42.7	90.7	133.4	100.0
	Test	32.1	54.1[b]	86.2[b]	85.0

[a] Average of five replicates.
[b] Significantly different from control at 0.05 level according to Student's *t* test.

TABLE 4. EFFECT OF DECAYING *E. prostrata* RESIDUES ON GERMINATION AND GROWTH OF TEST SPECIES

Test species	Treatments	Oven-dry weight (mg)[a]			Germination (% of Control)
		Root	Top	Seedling	
Cynodon dactylon	Control	14.1	21.2	35.3	100.0
	Test	4.5[b]	3.5[b]	8.0[b]	28.6
Amaranthus retroflexus	Control	44.4	39.5	83.9	100.0
	Test	8.1[b]	11.9[b]	20.0[b]	42.6
Gossypium hirsutum	Control	351.9	471.8	823.7	100.0
	Test	265.3	429.2	694.5	87.2
Medicago sativa	Control	12.3	33.4	45.7	100.0
	Test	7.4	28.5	35.9	76.9

[a] Average of five replicates.
[b] Significantly different from control at 0.05 level according to Student's *t* test.

and seedling growth of the other species. The aqueous extract appreciably inhibited root growth of *G. hirsutum* and *M. sativa* and appreciably stimulated root growth of *A. retroflexus*.

Residue Bioassay. Decaying residues of *E. prostrata* drastically reduced germination and growth of bermudagrass and *A. retroflexus* (Table 4), and germination and growth of the other test species were inhibited appreciably also.

Root Exudate Bioassay. Root exudates of *E. prostrata* significantly inhibited root, top, and seedling growth of bermudagrass and *A. retroflexus* and root

TABLE 5. EFFECT OF ROOT EXUDATES OF *E. prostrata* ON GROWTH OF TEST SPECIES

Test species	Treatments	Oven-dry weight (mg)[a]			Weight of seedlings as % of Control
		Root	Top	Seedling	
Cynodon dactylon	Control	25.0	46.9	71.9	100.0
	Test	6.5[b]	19.7[b]	26.2[b]	86.4
Amaranthus retroflexus	Control	63.2	409.0	472.2	100.0
	Test	27.6[b]	136.3[b]	163.9[b]	34.7
Gossypium hirsutum	Control	101.4	405.1	506.5	100.0
	Test	92.9	376.4	469.3	92.6
Medicago sativa	Control	17.5	45.8	62.8	100.0
	Test	7.8[b]	44.6	52.4	83.4

[a] Average of five replicates.
[b] Significantly different from control at 0.05 level according to Student's *t* test.

growth of *M. sativa* (Table 5). Top and seedling growth of *M. sativa* were appreciably inhibited.

DISCUSSION

The rapid invasion of *E. prostrata* into heavy sods of bermudagrass is apparently not due to differences in pH, electrical conductivity, minerals, and soil texture. Furthermore, the observed vegetational patterning could not be attributed to soil moisture since it commonly occurs in irrigated bermudagrass lawns and gardens. Light could not account for vegetational patterning either because *E. prostrata* is very small and does not intercept light appreciably. However, soil collected from *E. prostrata* stands at the end of its life cycle and stored until the beginning of the growing season significantly inhibited germination and growth of bermudagrass. This suggests that toxins released from *E. prostrata* remain stable long enough to affect growth of bermudagrass and probably explains why bermudagrass is not observed the next season in an area occupied by *E. prostrata*. The stability of phytotoxins released from some plant species has been reported by several workers (Alsaadawi and Rice, 1982; Achhireddy and Singh, 1984; Guenzi et al., 1967). It is possible that soil of bermudagrass that was used as control soil had some allelopathic activity, and this could have partially masked the allopathic activity of soil under *E. prostrata* (Horowitz and Friedman, 1971). Subsequent experiments indicated that the toxins are water soluble and can be released from *E. prostrata* by decomposition of plant residues and root exudates. It is important that a small amount of *E. prostrata* residue is quite enough to affect growth on bermudagrass significantly. The accumulation of these small amounts with time may be more detrimental to bermudagrass growth at the end of the season. Additionally, these amounts apparently remain active and prevent seed germination and growth of bermudagrass in *E. prostrata* stands next season.

Thus, it appears that allelopathy is the major factor responsible for the patterning of vegetation observed in this study. However, once germination and growth of bermudagrass are inhibited by allelopathic compounds released from *E. prostrata*, competition undoubtedly accentuates growth inhibition. Preliminary work indicated the aqueous extract of *E. prostrata* contained some inhibitory compounds of a phenolic nature.

Our results showed that *E. prostrata* is more allelopathic to bermudagrass and *A. retroflexus* than to *M. sativa* and cotton. This suggests that residues or living plants of *E. prostrata* may prove useful to control bermudagrass and *A. retroflexus* in certain crops. It would be premature to suggest specific methodology, however.

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GENETIC SOURCES OF PHEROMONE VARIATION IN *Colias eurytheme*[1] BUTTERFLIES

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Abstract—The courtship pheromone of *Colias eurytheme* butterflies varies greatly among males in both the quantities and relative proportions of its three chemical components [*n*-heptacosane (C27), 13-methylheptacosane (13MH), *n*-nonacosane (C29)]. Narrow-sense heritabilities were high for the blend of 13MH and C27 and for the component quantities in one population (Kansas) but were low for the other population tested (Arizona). Genetic correlations between the three components were high in both populations, indicating a substantial degree of additive genetic influence on the component blends. High variability among populations in phenotypic correlations suggests that much of the male-to-male variation in the courtship pheromone may be attributable to environmental or developmental sources. Pheromone phenotypes do not seem to be associated with alba genotype.

Key Words—*Colias*, Lepidoptera, Pieridae, courtship pheromone, pheromone variation, cuticular hydrocarbons, *n*-heptacosane, 13-methylheptacosane, *n*-nonacosane, heritability, genetic correlation, gas chromatography.

INTRODUCTION

Colias eurytheme (Pieridae) females are able to distinguish among conspecific and contraspecific males during courtship on the basis of male courtship pheromone and ultraviolet reflectance pattern on the wings (Silberglied and Taylor,

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1973, 1978; Taylor, 1973; Grula et al., 1980; Grula and Taylor, 1979, 1980). The pheromone is located on the dorsal surface of the hindwing (Silberglied and Taylor, 1978; Rutowski, 1980) and consists of *n*-heptacosane (C27), 13-methylheptacosane (13MH), and possibly *n*-nonacosane (C29) (Grula et al., 1980). These three chemicals have been identified together or in part in the epicuticular hydrocarbon fraction of many other species of insects including other lepidopterans (Grant et al., 1987; De Renobales and Blomquist, 1983; Jones et al., 1973). The use of such heavy, low-volatility compounds as short-range or contact pheromones is not unusual (Inscoe 1982) and can provide long-term efficacy without need for replenishment (Grula et al., 1980; Peschke and Metzler, 1987).

Multicomponent pheromones are common in many species of insects (Roelofs and Cardé, 1977; Silk and Kuenen, 1988), and differences in the relative quantities of the same components (i.e., blend differences) may serve as prereproductive isolating mechanisms among closely related species (Roelofs, 1979; Foster and Dugdale, 1988). However, variation in pheromone blend within a species could provide a substrate for intraspecific mate selection.

Females of the two cooccurring color morphs, orange and alba (white), impose centrifugal directional sexual selection on the pheromone blend of male *C. eurytheme* (Sappington, 1989; Sappington and Taylor, 1990a). It is suspected that much of the high variation in pheromone component quantities and blends within populations of this species (Sappington, 1989; data reported herein) is maintained in part by this disruptive selection.

To understand the evolutionary implications of such selection one must have some knowledge about the heritabilities of the pheromone characters involved and the genetic correlations between them (McCauley 1986). Fisher's Fundamental Theorem predicts that response to selection in a given generation is equal to the additive genetic variance present for fitness. Narrow-sense heritability (h^2) is the ratio of additive genetic variance to the phenotypic variance and determines, to a large extent, the degree of resemblance among relatives (Falconer, 1981). Because selection can only operate on additive genetic variance, h^2 can be used to predict the response to selection in a population. Good estimates of h^2 are notoriously difficult to obtain because of logistic difficulties and the necessity of often prohibitively large sample sizes to reduce the standard errors to reasonable levels. In addition, h^2 estimates are only valid for the population for which they are made (Falconer, 1981) and for the environment in which the population exists (McCauley, 1986). Various models of sexual selection predict or require that the trait(s) in question have either high or low heritabilities. However, as Endler (1986) emphasizes, it is a serious mistake to generalize an estimate of h^2 made in one location to other populations in other areas. Phenotypic selection could be uniform across all populations even though

heritabilities may be zero in some places and significantly greater than zero in others (Endler, 1986).

One would expect that if strict blend specificity of a pheromone is a component of reproductive fitness, either because of directional sexual selection or stabilizing selection, the amount of additive genetic variance for this character should be low (Robertson, 1955; Maynard Smith, 1985; Falconer, 1981). This expectation has been borne out in general for other characters associated with fitness (Roff and Mousseau, 1987; Mousseau and Roff, 1987; Gustafsson, 1986), but there are many exceptions (Mousseau and Roff, 1987). Estimates of heritability (h^2) for pheromone quantity or quality are rare. Collins and Cardé (1985) found the h^2 estimate of 0.342 ± 0.084 for pheromone component proportion in *Pectinophora gossypiella* to be slightly higher than expected. From selection experiments, Roelofs et al. (1986) calculated realized h^2 for a tortricid pheromone blend as 0.85. Collins et al. (1987) estimated h^2s for the quantities of 10 of 12 components of the alarm pheromone in honeybees (*Apis mellifera*). All estimates were high ($h^2 \geq 0.48$) but standard errors are very large and only the extremely high estimates for butyl acetate ($h^2 = 1.94 \pm 0.66$) and hexyl acetate ($h^2 = 1.98 \pm 0.66$) seem to be significantly different from zero. Du et al. (1987) measured the repeatability of pheromone blends in five females by analyzing pheromone composition on two different days in *Yponomeuta rorellus* and in 10 females of *Y. padellus*. Repeatability is a measure of the upper limit of h^2 for a character (Falconer, 1981) and ranged from 0.82 to 0.91 for all combinations except one (0.51).

Grula and Taylor (1979) demonstrated the broad-sense heritability of the pheromone in *C. eurytheme* by examining its production in progeny from interspecific crosses with the closely related sulfur, *C. philodice*. Analyses showed that most of the loci controlling the ratio of 13MH to C27 in *C. eurytheme* must be on the X chromosome. However, it is clear from their data that this character is multigenic and that there is some autosomal influence on the quantity of at least one of these two hydrocarbons. It is not clear whether the relative amounts of 13MH and C27 are specifically controlled by one or more loci, or if the ratio is a by-product of independent control of the quantities of the two constituents, because inheritance of the quantities was not examined. Inheritance of quantities and relative amounts of C29 was not investigated. It was not possible for Grula and Taylor (1979) to examine interindividual variation in pheromone component quantities and ratios, because the sensitivity of the available chromatographic equipment required the combined extraction of 20 individuals to obtain a readable output.

In this paper we describe the magnitude and form of variation in the male courtship pheromone of *Colias eurytheme* and report the results of studies investigating the degree to which this variation is genetically determined. Results of

studies on developmental and environmental sources of variation are reported elsewhere (Sappington and Taylor, 1990b).

METHODS AND MATERIALS

Pheromones of individual males were extracted by soaking both hindwings in about 1 ml hexane for 10 min. Scott (1986) has shown that soaking adult *D. melanogaster* in hexane for 1 min is enough to remove 95% of the cuticular hydrocarbons. Three microliters of an internal standard (*n*-octacosane at 1 μg/μl) were added to the extracts except in the first heritability experiment when 5 μl were added. The extracts were cleared of scales with a syringe filter, and concentrated to about 0.05–0.10 ml by evaporation under a gentle stream of nitrogen. Three microliters of concentrated extract were analyzed using a Hewlett-Packard 5790A series gas chromatograph equipped with a flame-ionization detector and interfaced with a H-P 3390A series integrator. A nonpolar, 25-m-long, bonded-phase, vitreous silica capillary column was used. It had a 0.22-mm inner diameter and film thickness of 0.25 μm. The carrier gas was helium, and flow rate was set at 25 cm/sec at an oven temperature of 250°C. The sample was split in the injector port so that 1% of the injected extract was swept into the column. Most samples were run isothermally at 250°C for 2 min postinjection, after which oven temperature was increased at 5°/min to 300°C and maintained at that temperature for 2 min. An exception was the first heritability study in which the temperature program started at 180°C.

Quantities (in micrograms) were estimated for *n*-heptacosane (C27), 13-methylheptacosane (13MH), and *n*-nonacosane (C29) by comparing the area under these peaks to the area under the internal-standard peak. Other quantities calculated were C27 and C29 combined (C27 + C29) and total pheromone (C27 + C29 + 13MH). Ratios of component peak areas were calculated for the following combinations: 13MH:C27, 13MH:C29, 13MH:(C27 + C29), C27:C29. Ratios are useful in that they provide a simultaneous measure of each component independent of overall increases or decreases in total quantity (Scott and Richmond, 1988). However, if the component in the numerator is smaller than the denominator, then all values for the ratio range between 0 and 1, while if the numerator is larger, it can range from 1 to infinity. Therefore, all ratios were $\log_{10}$-transformed.

Analyses were made of males collected from four locations in 1988: August 5–9 near El Centro, Imperial County, California; August 10–12 just east of Wellton, Yuma County, Arizona; August 13–15 near Antelope, Yuma County, Arizona; and September 6–14, 8 km east of Lawrence, Douglas County, Kansas. Samples from Lawrence were also taken in August and September of 1986 and 1987. The Arizona and California populations were in irrigated alfalfa fields

and are essentially 100% *C. eurytheme*, while the Lawrence population is sympatric with *C. philodice*. At the Wellton and Antelope sites, populations were so dense that random samples were taken by sweeping through the alfalfa canopy (20 strokes per sample) at different spots in the fields in the morning before mating activity began (before 1000 hr MST). The populations in El Centro and Lawrence were far less dense, and random samples were made by capturing the first 50 males seen on two different days. Samples were taken early in the morning while most males were preoccupied with feeding rather than searching for females. Mated pairs were collected as they were encountered.

A laboratory colony of *C. eurytheme* was begun in August 1985 and was maintained for 16 generations. The first generation was founded by eight wild females (two alba, six orange) that had already mated with eight unknown wild males. The females were collected in Lawrence and allowed to oviposit on sprigs of alfalfa. Larvae were reared on the bean diet described in Taylor et al. (1981) in 30-ml plastic cups with paper lids. Fifteen eggs were put in each cup, and larval density was later reduced to five or six larvae per cup. Pupae were refrigerated as needed to allow synchronization of adult emergence. Adults were marked as to brood and released into a 58-cm-W × 52-cm-L × 75-cm-H indoor flight cage. Butterflies were provided numerous artificial flowers from which to feed. Each consisted of a 3-ml shell vial filled with honey-water (one part honey to four parts distilled water) with a sponge inserted to soak up the fluid. Both were surrounded by interwoven red and yellow plsatic strips and were hung by wire along the top margins of the cage where the butterflies tended to congregate. Opportunity for mating was controlled by turning on or off four banks of overhead, heat-producing fluorescent lights. The cage was checked every half hour for mated pairs, which were removed and placed in individual cups; mated males were subsequently frozen. Mated females were fed and placed in individual oviposition cages. After seven days, unmated males were placed in envelopes and frozen. Several wild males were placed in the flight cage to mate with first generation females, three of which succeeded. After this, no new wild genes were introduced to the colony.

Narrow-sense heritabilities (h^2) were calculated for the nine pheromone characters described earlier for two populations using parent–offspring regression (Falconer, 1981). The pheromones of 28 fathers from the second laboratory generation, and three sons from each of these, were extracted and analyzed. Thirty-five mated pairs were captured in Antelope, Arizona in August 1988, and the females were fed and brought back alive to oviposit in the lab in Lawrence, 27 of which survived and produced fertile eggs. The sires were frozen after completion of copulation and the pheromones extracted along with those of one to five (depending on availability) male offspring reared on artificial diet.

It is possible that pheromone phenotype is correlated with alba genotype. *Colias* females occur in two color phases, orange or alba (white). This is thought

to be a simple sex-limited Mendelian character with the alba (*A*) allele dominant over the recessive orange (*a*) allele (Gerould, 1923). There are physiological and selective trade-offs associated with the alba polymorphism (Watt, 1973; Graham et al., 1980; Gilchrist and Rutowski, 1986), and some sort of assortative mating by females based on alba genotype of males may be advantageous. If so, females probably must rely on pheromonal cues because the alba genotype of males cannot be assessed visually due to the sex-limited nature of this trait.

To determine if alba genotype affects the pheromone phenotype, the pheromones of males of the eighth (C) and ninth (D) laboratory generations were analyzed and compared across groups of alba genotypes. Males from 0 : 1 [alba (A) : orange (O)] broods were presumably *aa*; males from 1 : 1 broods are either *aa* or *Aa*; and males from 1 : 0 broods are either *Aa* or *AA*. Males from 3 : 1 broods can be either *aa*, *Aa*, or *AA* and are excluded from the analysis. The two generations were analyzed separately. Both C27 and C29 occur on female wings as well (Grula et al., 1980; and this study), and quantities and relative quantities for these two hydrocarbons on alba and orange females were compared using butterflies from the Wellton and Antelope, Arizona, populations.

TABLE 1. MEANS + SD (*N*) OF PHEROMONE COMPONENT QUANTITIES AND Log_{10} RATIOS OF ALL MALES ANALYZED (MATED, RANDOM, AND/OR UNMATED CLASSES COMBINED)

		Mean ± SD (μg)			Ratio
Population	Year	C27	13MH	C29	13MH/C27
El Centro, California	1988	0.57 ± 0.557(99)	1.55 ± 1.223(99)	3.09 ± 2.391(99)	0.47 ± 0.255(99)
Antelope, Arizona	1988	0.76 ± 0.629(150)	3.04 ± 1.848(150)	3.72 ± 2.79(150)	0.65 ± 0.300(150)
Wellton, Arizona	1988	0.79 ± 0.620(144)	2.41 ± 2.206(144)	3.91 ± 3.389(144)	0.51 ± 0.254(144)
Lawrence, Kansas	1988	0.57 ± 0.721(72)	3.63 ± 2.986(72)	3.86 ± 2.664(72)	0.88 ± 0.360(72)
Lab C generation	1986	3.14 ± 1.336(42)	2.89 ± 1.582(42)	3.02 ± 0.960(42)	−0.07 ± 0.268(42)
Lab D generation	1986	3.30 ± 0.991(37)	1.51 ± 0.963(37)	2.87 ± 0.826(37)	−0.44 ± 0.396(37)
Lab E generation	1987	2.56 ± 0.892(18)	1.25 ± 0.630(18)	2.40 ± 0.583(18)	−0.34 ± 0.285(18)
Lab G generation	1987	2.02 ± 0.729(39)	1.84 ± 0.989(39)	2.14 ± 0.824(39)	−0.09 ± 0.299(39)

RESULTS

High between-male variation in both pheromone component quantities and component ratios was characteristic of all populations of *Colias eurytheme* examined (Table 1, Figures 1–3) as well as among males used in the two estimates of h^2 (Tables 2 and 3). Heritabilities and their associated 95% confidence intervals were calculated by parent–offspring regressions, where $h^2 = 2b$ with one parent (Falconer, 1981), for the second generation Kansas lab colony and for the Antelope, Arizona, population (Tables 2 and 3). For the lab colony, the h^2 estimates for quantity and 13MH:C27 are significantly different from zero, and of these, C29, 13MH, and 13MH:C27 have very high values (Table 2). However, for the Arizona population, all h^2 estimates are low, except for C27:C29, which has a large standard error, and none are significantly different from zero.

Genetic correlations (r_A) (Falconer, 1981) between the component quantities were calculated for the two populations for which father and son data were available (Table 4). All correlations were very high, and all were significantly

TABLE 1. CONTINUED

Ratio				
13MH/C29	13MH/(C27 + C29)	C27/C29	C27 + C29	Total (μg)
−0.27 ± 0.291(99)	−0.36 ± 0.257(99)	−0.75 ± 0.298(99)	3.67 ± 2.767(99)	5.22 ± 3.772(99)
−0.04 ± 0.348(150)	−0.14 ± 0.301(150)	−0.69 ± 0.353(150)	4.48 ± 3.125(150)	7.53 ± 4.212(150)
−0.19 ± 0.249(144)	−0.29 ± 0.218(144)	−0.70 ± 0.301(144)	4.71 ± 3.723(144)	7.12 ± 5.694(144)
−0.03 ± 0.293(72)	−0.10 ± 0.280(72)	−0.91 ± 0.336(72)	4.43 ± 3.129(72)	8.05 ± 5.664(72)
−0.06 ± 0.241(42)	−0.37 ± 0.247(42)	0.00 ± 0.112(42)	6.15 ± 2.163(42)	9.04 ± 3.090(42)
−0.39 ± 0.396(37)	−0.72 ± 0.391(37)	0.06 ± 0.113(37)	6.18 ± 1.607(37)	7.69 ± 2.054(37)
−0.33 ± 0.272(18)	−0.64 ± 0.272(18)	0.01 ± 0.118(18)	4.96 ± 1.350(18)	6.22 ± 1.618(18)
−0.11 ± 0.330(39)	−0.41 ± 0.305(39)	−0.02 ± 0.166(39)	4.17 ± 1.335(39)	6.01 ± 1.707(39)

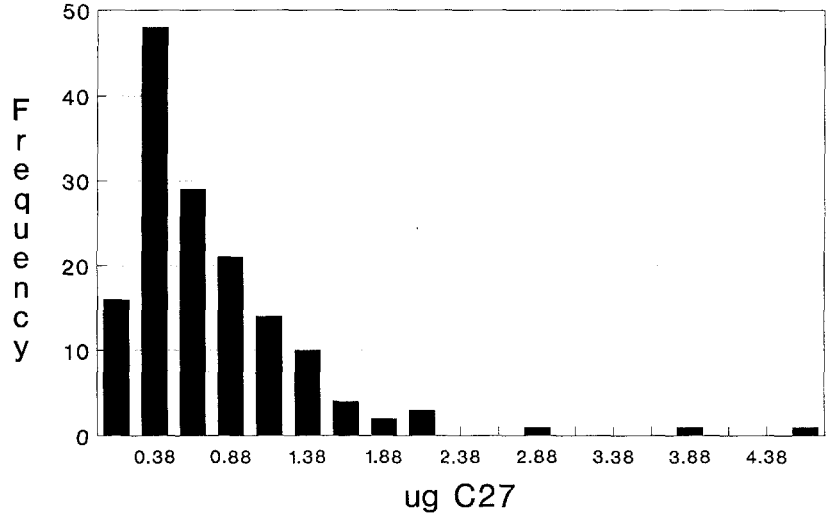


FIG. 1. Frequency distribution of μg C27 on hindwings of males captured in sweep-net samples and in copula with alba and orange females in Antelope, Arizona, August 1988.

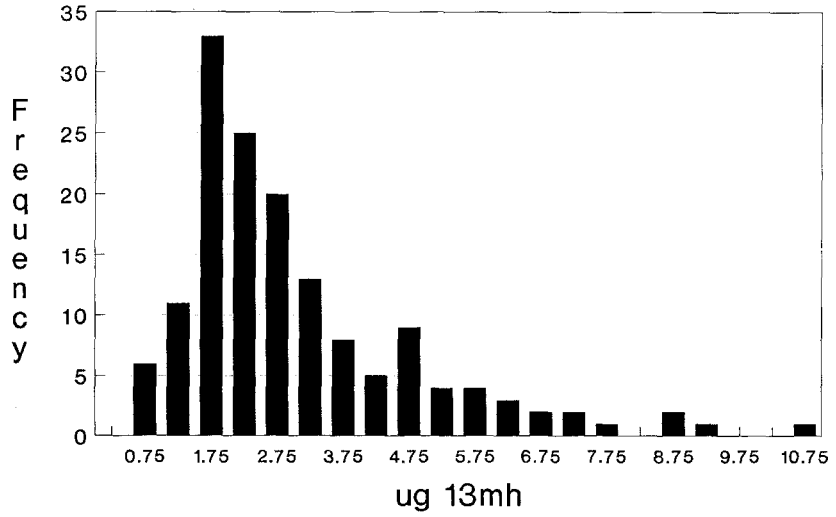


FIG. 2. Frequency distribution of μg 13MH on hindwings of males captured in sweep-net samples and in copula with alba and orange females in Antelope, Arizona, August 1988.

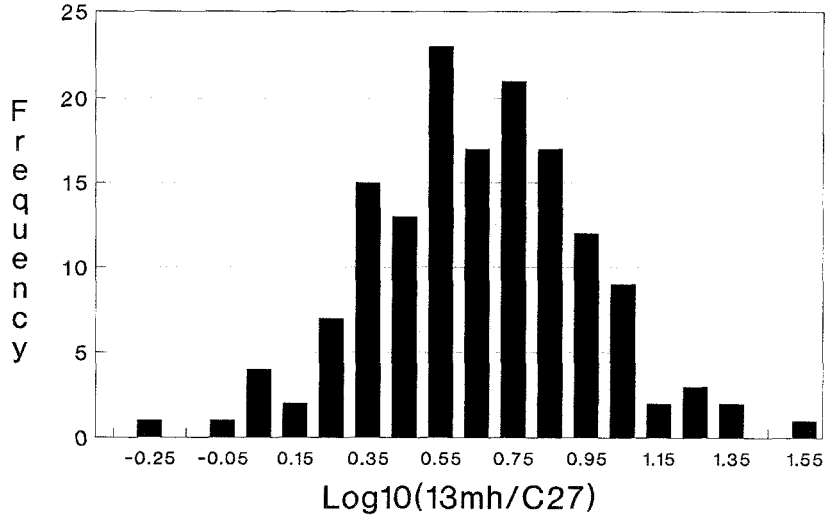


FIG. 3. Frequency distribution of $\log_{10}$(13MH/C27) on hindwings of males captured in sweep-net samples and in copula with alba and orange females in Antelope, Arizona, August 1988.

different from zero. Phenotypic correlations between the three components were calculated for 10 different populations (Table 5). Although many of the correlations were significant, some were not, and their magnitude varied greatly between populations.

Pheromones of laboratory males from 0:1, 1:1, and 1:0 (alba:orange) broods were compared in an effort to detect differences in composition that may be associated with alba genotypes (Table 6). In the C generation, low quantities of 13MH and low 13MH:*n*-alkane ratios are associated with the *aa* genotype, but this relationship is not seen in the D generation. Likewise, comparisons of C27 and C29 on alba and orange female wings (females lack detectable quantities of 13MH) show a large difference in one population but not the other (Table 7). The reasons for these discrepancies are not clear.

DISCUSSION

Genetic correlations (r_A) (Falconer, 1981) measure the extent to which two measurements represent the same character, so high genetic correlations between two pheromone components in *P. gossypiella* (Collins and Cardé 1985) and

TABLE 2. HERITABILITY (h^2) ESTIMATES FOR PHEROMONE COMPONENT QUANTITIES AND Log_{10} RATIOS FOR SECOND GENERATION LAB-REARED MALES, LAWRENCE, KANSAS STOCK, 1985

	μgC27	μg13MH	μgC29	13MH/C27	13MH/C29	13MH/(C27 + C29)	C27/C29	μg(C27 + C29)	μgTotal
N	28	28	28	29	29	29	29	28	28
Sire mean	2.18	5.02	0.45	0.40	0.32	0.04	−0.06	4.80	9.82
Sire s^2	0.75	2.82	1.62	0.04	0.04	0.04	0.02	3.97	10.26
Mid-son mean	1.03	3.86	1.93	0.55	0.28	0.10	−0.26	2.96	6.81
Mid-son s^2	0.26	2.98	0.92	0.03	0.02	0.03	0.003	2.12	8.36
h^2	0.20	0.89	0.81	0.89	0.31	0.05	0.14	0.62	0.28
95% CI for h^2	0–0.67	0.14–1	0.29–1	0.29–1	0–1	0–0.79	0–0.34	0.09–1	0–1

TABLE 3. HERITABILITY (h^2) ESTIMATES FOR PHEROMONE COMPONENT QUANTITIES AND Log_{10} RATIOS FOR ANTELOPE, ARIZONA, 1988

				Ratio					
	μgC27	μg13MH	μgC29	13MH/C27	13MH/C29	13MH/(C27 + C29)	C27/C29	μg(C27 + C29)	μgTotal
N	27	27	27	27	27	27	27	27	27
Sire mean	0.40	1.43	2.38	0.68	−0.15	−0.22	−0.81	2.78	4.21
Sire s^2	0.24	0.35	6.26	0.08	0.06	0.06	0.04	8.80	11.62
Mid-son mean	0.48	2.37	2.87	0.73	−0.07	−0.14	−0.73	3.37	5.74
Mid-son s^2	0.02	0.44	0.86	0.01	0.02	0.01	0.13	0.99	2.27
h^2	−0.75	−0.73	−0.12	0.00	0.04	0.03	0.59	−0.12	−0.19
95% CI for h^2	0–0.17	0–0.16	0–0.19	0–0.31	0–0.47	0–0.41	0–1	0–0.16	0–0.17

TABLE 4. GENETIC CORRELATIONS (r_A) AND PHENOTYPIC CORRELATIONS (r_P) BETWEEN PHEROMONE COMPONENT QUANTITIES

	C27 vs 13MH	C29 vs 13MH	C27 vs C29
Lawrence, Kansas ($N = 28$) r_A	0.84	0.78	0.98
95% CI of r_A	0.81–0.87	0.66–0.89	0.96–0.99
Antelope, Arizona ($N = 27$) r_A	0.82	0.86	1.04
95% CI of r_A	0.13–1.0	0.55–1.0	0.92–1.17
r_P (sons)	0.59	0.70	0.53
Prob $> r_P^a$	0.0001	0.0001	0.0001

[a] Under H_0: Rho = 0.

TABLE 5. PHENOTYPIC CORRELATIONS BETWEEN PHEROMONE COMPONENT QUANTITIES

	Year	N	C27 vs 13MH	C29 vs 13MH	C27 vs C29
Lawrence, Kansas	1986	43	−0.16	0.32[a]	0.22
Lawrence, Kansas	1987	80	−0.16	0.41[c]	0.46[c]
Lawrence, Kansas	1988	53	0.64[c]	0.68[c]	0.60[c]
El Centro, California	1988	99	0.67[c]	0.71[c]	0.61[c]
Antelope, Arizona	1988	97	0.32[b]	0.27[a]	0.38[c]
Wellton, Arizona	1988	79	0.46[c]	0.87[c]	0.46[c]
Lab C generation	1986	42	0.24	0.44[b]	0.77[c]
Lab D generation	1986	38	0.24	0.19	0.58[c]
Lab E generation	1987	18	0.30	0.08	0.66[b]
Lab G generation	1987	83	0.44[c]	0.57[c]	0.83[c]

[a] Significant correlation at 0.05 level.
[b] Significant correlation at 0.01 level.
[c] Significant correlation at 0.0001 level.

between many of the components of the honeybee alarm pheromone (Collins et al., 1987) indicate that ratios of these components are valid characters. Sanders et al. (1977) found that F_1 hybrids of two sibling species of *Choristoneura* (Tortricidae) produce pheromone blends that attract males of one or the other parental species but not both, indicating that the blends are under genetic regulation (Silk and Keunen, 1988). In *Drosophila melanogaster*, a component of the female's epicuticle, 7,11-heptacosadiene, elicits courtship behavior in males

TABLE 6. COMPARISON OF QUANTITIES AND Log_{10} RATIOS OF PHEROMONE COMPONENTS FROM MALES FROM DIFFERENT ALBA–ORANGE BROODS FROM TWO LABORATORY GENERATIONS, LAWRENCE, KANSAS 1986 (MEANS ± SE)

			Amount (μg)			Ratio					
Generation	*N*	A:O	C27	13MH	C29	13MH/ C27	13MH/ C29	13MH/ (C27 + C29)	C27/C29	C27 + C29 (μg)	Total (μg)
C	7	0:1	1.14 ± 0.09	0.55 ± 0.10	1.12 ± 0.10	−0.36 ± 0.11	−0.36 ± 0.12	−0.82 ± 0.22	0.01 ± 0.02	2.25 ± 0.19	2.80 ± 0.15
C	19	1:1	0.99 ± 0.12	1.05 ± 0.14	0.97 ± 0.08	−0.01 ± 0.06	−0.01 ± 0.05	−0.31 ± 0.05	−0.01 ± 0.03	1.96 ± 0.19	3.01 ± 0.28
C	11	1:0	1.08 ± 0.14	1.01 ± 0.12	0.99 ± 0.10	−0.02 ± 0.05	0.00 ± 0.04	−0.31 ± 0.04	0.03 ± 0.04	2.07 ± 0.23	3.08 ± 0.32
D	10	0:1	3.65 ± 0.35	1.86 ± 0.30	2.81 ± 0.22	−0.33 ± 0.08	−0.23 ± 0.08	−0.58 ± 0.08	0.11 ± 0.02	6.45 ± 0.56	8.31 ± 0.63
D	10	1:1	3.48 ± 0.30	1.33 ± 0.28	2.88 ± 0.26	−0.50 ± 0.09	−0.42 ± 0.08	−0.77 ± 0.08	0.08 ± 0.02	6.36 ± 0.53	7.69 ± 0.73
D	12	1:0	2.91 ± 0.27	1.70 ± 0.30	2.94 ± 0.33	−0.31 ± 0.10	−0.31 ± 0.10	−0.61 ± 0.10	0.00 ± 0.04	5.85 ± 0.52	7.49 ± 0.64
Kruskal-Wallis tests: P =											
C			0.44	0.04	0.47	0.03	0.02	0.01	0.71	0.34	0.84
D			0.20	0.41	0.98	0.34	0.29	0.35	0.17	0.80	0.53

TABLE 7. COMPARISONS OF QUANTITIES AND Log_{10} RATIO OF C27 AND C29 ON WINGS OF ALBA AND ORANGE FEMALES (MEAN ± SE)

Population	Phenotype	*N*	C27 (μg)	C29 (μg)	C27/C29	C27 + C29 (μg)
Wellton	Alba	39	2.56 ± 0.281	2.64 ± 0.120	−0.04 ± 0.030	5.20 ± 0.342
	Orange	40	2.64 ± 0.216	2.62 ± 0.165	0.00 ± 0.037	5.26 ± 0.298
P <			0.78	0.54	0.70	0.88
Antelope	Alba	47	2.81 ± 0.187	2.16 ± 0.131	0.11 ± 0.021	4.98 ± 0.296
	Orange	50	1.94 ± 0.095	1.79 ± 0.106	0.04 ± 0.027	3.73 ± 0.171
P <			0.0001	0.021	0.011	0.0002

(Antony and Jallon, 1982; Antony et al., 1985) and is found in different amounts in different strains of *D. melanogaster* (Antony et al., 1985; van den Berg et al., 1984; Jallon, 1984; Luyten, 1983). In addition, males are characterized by two cuticular pheromones, 7-tricosene and 7-pentacosene (Antony and Jallon, 1982; Antony et al., 1985), that stimulate the female to mate, and the ratio between them varies with the strain. Scott and Richmond (1988) found that the quantities of these two components are under the control of unlinked autosomal genes with some influence from the X chromosome but that the relative amounts seem to be controlled by linked autosomal loci.

Genetic correlations between the pheromone components of *C. eurytheme* indicate a high degree of additive genetic influence on the component ratios (Table 5), and one must conclude that the component ratios are valid characters (Collins and Cardé, 1985) rather than the simple products of independently controlled component quantities. Phenotypic correlations are often fair estimators of genetic correlations (Cheverud, 1988), but the broad range of phenotypic correlations among several populations of *C. eurytheme* (Table 6) suggests that environmental or developmental factors can affect individual component quantities differently, resulting in an increase in ratio variability. For example, although the estimates for the genetic correlation between C27 and C29 quantities (Table 5) indicate that these two characters are controlled by the same set of genes, nongenetic factors can affect the quantities of these characters to such an extent that the phenotypic correlation between them is greatly reduced or even lost altogether (e.g., Lawrence, Kansas, 1986; Antelope, Arizona 1988; Table 6).

The low h^2s in one of the two populations tested (Table 3) and the high variation in phenotypic correlations (Table 6), in spite of high genetic correlations between component quantities (Table 5), suggests that the high degree of pheromone variability in *C. eurytheme* populations (Table 1, Figures 1–3) is caused in part by environmental and/or developmental factors. Some of these

potential sources are examined in Sappington and Taylor (1990b). Because extrapolation of h^2 estimates from one population to another is inappropriate (Falconer, 1981; Endler, 1986), the specific magnitudes of genetic contributions to the observed variation remain obscure. However, our data show (Table 2) that significant additive genetic variance for pheromone component quantities and blends does exist, at least in some populations, suggesting that response to the observed disruptive sexual selection (Sappington, 1989; Sappington and Taylor, 1990a) is likely contributing to the high variability of these characters in this species.

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DEVELOPMENTAL AND ENVIRONMENTAL SOURCES OF PHEROMONE VARIATION IN *Colias eurytheme* BUTTERFLIES[1]

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Abstract—Body size, age, ambient temperature, wing wear, and flight activity were investigated as possible sources of variation in the quantities and relative proportions of the three chemical components [*n*-heptacosane (C27), 13-methylheptacosane (13MH), and *n*-nonacosane (C29)] of the male courtship pheromone of *Colias eurytheme* butterflies. Size of the male has very little influence on the amount of any of the pheromone components present on the wings. Most of the deposition of all three components onto the surface of the hindwing occurs between 3 and 9 hr after emergence from the pupa. 13MH is deposited more rapidly than C27 and C29, and C27 more rapidly than C29. After the first 12 hr posteclosion, the pheromone phenotype of an individual male remains relatively constant through at least 96 hr of age. Experiments showed that none of the three chemicals volatilize to any appreciable extent at temperatures likely to be experienced in the field. The pheromones of actively searching and courting males did not differ from those of less-active feeding and resting males, suggesting that volatilization induced by flight activity is not an important source of pheromone variation. Loss of scales with age does seem to affect pheromone phenotype, but not in a readily interpretable way. Although the quantity of 13MH was lower in worn males than in fresh, C27 was higher.

Key Words—*Colias*, Lepidoptera, Pieridae, courtship pheromone, pheromone variation, pheromone production, cuticular hydrocarbons, *n*-heptacosane, 13-methylheptacosane, *n*-nonacosane.

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INTRODUCTION

Female *Colias eurytheme* (Pieridae) are able to recognize conspecific males using both ultraviolet reflectance patterns and the male courtship pheromone as cues (Silberglied and Taylor, 1973, 1978; Taylor, 1973). The pheromone is located on the dorsal surface of the hindwing (Silberglied and Taylor, 1978; Rutowski, 1980) and consists of *n*-heptacosane (C27), 13-methylheptacosane (13MH), and possibly *n*-nonacosane (C29) (Grula et al., 1980). These hydrocarbons are found commonly in the epicuticular wax layer of many different insects (Sappington, 1989). Surveys of pheromone composition of males from several populations of *C. eurytheme* have shown that both the quantities and relative proportions (blends) of the three components vary greatly among individuals (Sappington, 1989; Sappington and Taylor, 1990a). Disruptive sexual selection on the courtship pheromone imposed by the two female morphs (orange and alba) (Sappington, 1989; Sappington and Taylor, 1990a) is probably responsible for maintaining some of the observed variation, but part of it derives from developmental and/or environmental sources (Sappington and Taylor, 1990a,b).

Many developmental sources of variation in pheromones and hydrocarbons have been identified in various insects. Age is frequently cited. In the rice stem borer, *Chilo suppressalis* (Pyralidae), quantities of the female pheromone decrease daily over the first three days posteclosion (Usui et al., 1988). The amount of pheromonally active (*Z*,*Z*)-9,19-alkadienes in female *Pikonema alaskensis* (Hymenoptera) increased from 10.2 μg at eclosion to 25.0 μg at 5 days old, while the amount of (*Z*,*Z*)-9,19-dienes remained constant with age (Bartelt et al., 1984). Young male rove beetles (*Aleochara curtula*) have hydrocarbon profiles similar to those of females and are therefore unattractive to them (Peschke, 1987). Antony and Jallon (1982) found that the level of surface hydrocarbons remain constant in virgin females from 3 to 5 days old in *Drosophila melanogaster*. In *D. virilis*, the quantity of hydrocarbons increases over the first four days of adulthood in both sexes and continues to increase to 8 days of age (Jackson and Bartelt, 1986). In addition, the average chain length of hydrocarbons decreases as the flies age, and two hydrocarbons change differentially depending on sex after 4 days of age. Other *Drosophila* species (Bartelt et al., 1986), the fleshfly *Sarcophaga bullata* (Jackson et al., 1974), and the milkweed bug *Oncopeltus fasciatus* (Jackson, 1983) also show age-related changes in hydrocarbon profiles. Kuwahara (1979) presented evidence that the ratio of the two major components of the courtship pheromone of *Pieris melete* varies with age of the individual.

Body size is not correlated with pheromone component quantities in *Chilo suppressalis* (Usui et al., 1988) or in *Aleochara curtula* (Coleoptera) (Peschke, 1987). However, Anderbrant et al. (1985) suggested that differing degrees of larval competition in *Ips typographus* (Coleoptera) may contribute to intraspe-

cific variation in the quality and quantity of the aggregation pheromone through its effects on the weight and fat content of the emerging adults.

It is possible for temperature to differentially affect the rate of volatilization of various components of a surface pheromone. For example, female rove beetles (*Aleochara curtula*) maintained at 27°C for 14 days show a greater loss of cuticular alkenes than of saturated hydrocarbons (Peschke, 1987).

In this study, we examined the effects of age, body size, ambient temperature, wing wear, and flight activity on the pheromone phenotypes of male *C. eurytheme*.

METHODS AND MATERIALS

Pheromones of individual males were extracted with hexane and were analyzed using gas chromatography. Procedural details may be found in Sappington and Taylor (1990a). The pheromone characters quantified for each male included micrograms of C27, 13MH, and C29; C27 and C29 combined (C27 + C29); total pheromone (C27 + 13MH + C29); and the ratios 13MH:C27, 13MH:C29, 13MH:(C27 + C29), and C27:C29, which were $\log_{10}$-transformed for the reasons presented in Sappington and Taylor (1990a).

Size of a male may influence the quantity of pheromone on the wings. Forewing length in *Colias* is correlated with dry weight (Gray 1967a, b) and was used as an index of body size. Regressions of pheromone characters on size and calculation of coefficients of determination (r^2) were performed for males collected randomly or in mated pairs from several populations (dates and locations of modern samples are described in Sappington and Taylor, 1990a) and from a laboratory colony (Sappington and Taylor, 1990a). Populations from Orchard Lake, Minnesota, Crystal Lake, Minnesota, and Tempe, Arizona, were sampled in 1966 and 1967 (see Taylor 1972 for specific dates and sampling methods). These samples have been stored in plastic bags in large cardboard drums during the intervening years, and little volatilization or degradation of the pheromones has occurred (Sappington, 1989). This is not too surprising given the high molecular weight of the chemicals involved and their low volatilities (Grula et al., 1980; data herein), nor is such long-term stability unprecendented (Carlson and Brenner, 1988; Huyton et al., 1980).

Two different experiments were performed to determine if pheromone quantity or quality change with age of the male. Laboratory-reared males were kept in small cylindrical (80-mm-diam. × 100-mm-tall) eclosion cages made of plastic screening, where flight activity was restricted, and allowed to age unfed for 1, 8, 12, 24, 48, 72, or 96 hr. Pheromones then were extracted and analyzed. In the second test, males were allowed to age a certain number of hours, after which a single hindwing was removed with scissors and the pheromone immediately extracted. The male was then returned to its cage to age a specified number of hours further before the other wing was removed and

extracted. The paired age groups were 1–3, 3–6, 6–9, 9–12, 12–16, 16–24, 24–48, 24–72, 48–96, and 144–192 hr. Amputation caused no obvious harm except possibly in 1-hr-old butterflies in which drops of hemolymph occasionally exuded from cut wing veins.

Males collected in Lawrence, Kansas, in 1986 and 1987 during unrelated experiments were classified by wing wear as either fresh (little or no scale loss) or worn (moderate to heavy scale loss). Pheromone component quantities and ratios were compared between the groups to determine if loss of scales causes a loss of pheromone as well.

We determined if ambient temperature causes volatilization of the pheromone components, and if so, whether they volatilize at different rates. The *n*-alkanes C27 and C29 are solids at room temperature, while 13MH is a liquid (Grula et al., 1980), but this does not necessarily mean that 13MH is more volatile than the solids (R. Carlson, University of Kansas, personal communication). Two tests were conducted in which one wing from each experimental male (lab-reared) was removed and the pheromone extracted, while the other wing was exposed to a heat-producing light bulb. In the first test, the wings were exposed for 3 hr at 38°C, while in the second test they were exposed for 24 hr at 54°C. There was no air movement over the wings during this time.

It is known that males spend most of their time seeking females by flying low over the vegetation and inspecting orange, yellow, or white objects (Taylor, 1972; Graham et al., 1980). It is also known, however, that not all males are as active as others, due in part to their PGI genotypes (Watt et al., 1985). We hypothesized that if pheromones, or parts of the pheromone, volatilize more readily during periods of flight activity (e.g., because of increased air flow), then males that are out searching for and courting females should have different pheromone compositions than males that are resting or feeding. On three consecutive days in August 1987 in a weedy alfalfa field in Lawrence, Kansas, 8–12 dead females were pinned to dowel sticks painted green (Silberglied and Taylor, 1978). They were placed in a natural resting position with wings closed and were exposed just above the alfalfa canopy where they were clearly visible to searching males. Males that approached and courted one or more of the dead females were captured and the pheromones compared to those of males captured while resting or feeding at flowers during the same time of day (1200–1500 hr CST). Ambient temperatures during the periods of collection ranged between 32 and 38°C.

RESULTS

Size of a male does not seem to be an important factor in determining its pheromone phenotype. Regressions of pheromone component quantities on forewing length (Table 1) show that only occasionally is a character signifi-

TABLE 1. REGRESSION COEFFICIENTS (b) AND COEFFICIENTS OF DETERMINATION (r^2) FOR PHEROMONE COMPONENT QUANTITIES REGRESSED ON FOREWING LENGTHS

			μgC27		μg13 MH		μgC29		μg(C27 + C29)		μg Total	
Population	Year	N	b	r^2	b	r^2	b	r^2	b	r^2	b	r^2
Orchard Lake, Minnesota	1966	78	0.02	0.00	0.29[a]	0.08	0.12	0.02	0.14	0.01	0.43	0.04
Crystal Lake, Minnesota	1966	146	0.03	0.01	0.02	0.01	0.20	0.01	0.22	0.01	0.10	0.02
Tempe, Arizona	1967	65	0.05	0.01	0.06	0.01	0.16	0.01	0.21	0.01	0.27	0.01
Antelope, Arizona	1988	97	0.01	0.00	0.19	0.21	0.27	0.01	0.28	0.02	0.48	0.02
Antelope, Arizona (lab-reared sons)	1988	110	0.02	0.01	0.14	0.03	0.12	0.01	0.14	0.01	0.28	0.02
Lawrence, Kansas	1987	80	0.03	0.00	0.62[b]	0.20	0.32[b]	0.17	0.36[a]	0.09	0.98[b]	0.02
Lawrence, Kansas	1988	65	0.03	0.01	0.43[a]	0.06	0.21	0.02	0.24	0.02	0.67	0.04

[a] Significant at 0.05 level.
[b] Significant at 0.01 level.

cantly related to size and even then the amount of variation accounted for by wing length is slight (r^2s < 0.25). Ratios of component quantities were only rarely related to wing length and never explained more than 7% of the variance.

It is not known whether *Colias* males secrete pheromone components throughout their life or if production is shut down soon after eclosion. In a preliminary test, pheromones from males of seven age classes were analyzed and compared (Table 2). A Kruskal-Wallis test across all age classes indicated that at least one age class differed significantly from the others for all pheromone characters except $\log_{10}$ (13MH : C29). Removal of the 1 hr age class from the test resulted in no significant differences, suggesting that an individual's pheromone phenotype stabilizes after the first 12 hr posteclosion.

The second age test involved comparing the pheromone on one wing removed from an individual at one age with that on the other wing removed at a different age. The means for each of 12 age classes ranging from 1 hr to 192 hr are presented in Table 3. As in the first test, pheromone phenotypes seem to stabilize by 9–12 hr posteclosion. The mean quantities of the three components seem much greater in the 144-hr and 192-hr age classes, but sample sizes are very small for these groups and the standard errors are quite large. The paired-comparison t tests (Table 4) indicate that at the level of individual butterflies, most of the differences between age classes in butterflies greater than 9 hr of age (Table 3) are not real, although again the sample size for the oldest age classes is very small. One exception is the significant increase in micrograms of C29 between 24 and 48 hr of age. Phenotypic correlations among component quanitities also vary with age (Table 5), peaking at age 9 hr and decreasing thereafter, suggesting differential environmental effects on pheromone components that accumulate with age.

Although age per se does not greatly affect pheromone phenotypes after the first 9–12 hr posteclosion, older males in natural populations may have different pheromone configurations than young males because of loss of scales with age. Rutowski (1985) found that an increase in wing wear causes a decrease in ultraviolet reflectance, and loss of pheromone-bearing scales from the dorsal surface of the hindwing also can be presumed. If pheromone components are not replenished, then one would expect a decrease in component quantities with an increase in wing wear. Males collected in Lawrence, Kansas, in 1986 and 1987 were classified as fresh or worn and pheromone characters were compared with a Mann-Whitney U test for both years (Table 6). As expected, worn males have less 13MH than fresh males, but, surprisingly, worn males have significantly more C27 than young males, and there is no difference in the quantity of C29 present. Thus, these age-related differences must be associated with some factor other than simple scale loss. These differential changes are reflected in the general breakdown of phenotypic correlations among component quantities as males become worn (Table 7), although this also could be related to male age per se (Table 5).

TABLE 2. MEANS ± SE FOR PHEROMONE QUANTITIES AND LOG_{10} RATIOS FROM FIRST AGE TEST USING MALES FROM LAB COLONY (G GENERATION)

Age (hr)	N	Amount (μg)			13MH/C27	13MH/C29	13MH/(C27 + C29)	C27/C29	C27 + C29 (μg)	Total (μg)
		C27	13MH	C29						
1	12	0.70 ± 0.13	0.27 ± 0.07	1.00 ± 0.27	−0.43 ± 0.08	−0.53 ± 0.11	−0.81 ± 0.09	−0.10 ± 0.10	1.71 ± 0.31	1.98 ± 0.33
8	2	1.45 ± 0.25	0.97 ± 0.23	2.47 ± 0.49	−0.18 ± 0.3	−0.04 ± 0.02	−0.61 ± 0.02	−0.23 ± 0.01	3.92 ± 0.74	4.89 ± 0.97
12	8	2.13 ± 0.37	3.51 ± 1.09	3.19 ± 0.67	0.07 ± 0.13	−0.07 ± 0.11	−0.31 ± 0.12	−0.14 ± 0.03	5.32 ± 1.04	8.83 ± 2.04
24	14	2.06 ± 0.29	2.53 ± 0.47	3.21 ± 0.40	0.05 ± 0.08	−0.16 ± 0.09	−0.37 ± 0.09	−0.20 ± 0.02	5.26 ± 0.68	7.79 ± 0.94
48	14	2.11 ± 0.35	2.86 ± 0.63	2.95 ± 0.53	0.03 ± 0.09	−0.08 ± 0.94	−0.33 ± 0.09	−0.12 ± 0.03	5.05 ± 0.88	7.91 ± 1.38
72	12	2.79 ± 0.41	2.93 ± 0.74	3.87 ± 0.82	−0.09 ± 0.10	−0.20 ± 0.08	−0.46 ± 0.09	−0.11 ± 0.04	6.66 ± 1.20	9.59 ± 1.70
96	4	2.73 ± 0.50	3.73 ± 1.87	2.89 ± 1.12	−0.08 ± 0.21	−0.20 ± 0.16	−0.45 ± 0.18	−0.12 ± 0.05	6.62 ± 1.61	10.4 ± 3.45

TABLE 3. MEANS ± SE FOR PHEROMONE QUANTITIES AND LOG_{10} RATIOS FROM SECOND AGE TEST USING MALE OFFSPRING OF WILD FEMALES

Age (hr)	N	Amount (μg) C27	13MH	C29	13MH/C27	13MH/C29	13MH/(C27 + C29)	C27/C29	C27 + C29 (μg)	Total (μg)
1	12	0.52 ± 0.09	0.29 ± 0.12	0.30 ± 0.06	−0.44 ± 0.23	0.28 ± 0.22	−0.76 ± 0.18	0.16 ± 0.19	0.82 ± 0.14	1.11 ± 0.22
3	22	0.46 ± 0.03	0.31 ± 0.03	0.42 ± 0.07	−0.19 ± 0.05	−0.03 ± 0.11	−0.46 ± 0.06	0.16 ± 0.09	0.88 ± 0.08	1.20 ± 0.90
6	19	0.70 ± 0.09	0.60 ± 0.09	0.76 ± 0.07	−0.07 ± 0.05	−0.13 ± 0.05	−0.42 ± 0.04	−0.07 ± 0.06	1.46 ± 0.13	2.06 ± 0.20
9	20	1.62 ± 0.56	2.01 ± 1.0	1.49 ± 0.51	−0.03 ± 0.07	−0.02 ± 0.07	−0.33 ± 0.07	0.00 ± 0.05	3.11 ± 1.1	5.12 ± 2.1
12	26	0.84 ± 0.12	0.91 ± 0.14	0.91 ± 0.10	−0.03 ± 0.10	−0.10 ± 0.09	−0.39 ± 0.09	−0.08 ± 0.06	1.75 ± 0.18	2.67 ± 0.28
16	26	1.31 ± 0.28	1.03 ± 0.15	1.47 ± 0.22	−0.06 ± 0.08	−0.17 ± 0.08	−0.43 ± 0.07	−0.12 ± 0.06	2.81 ± 0.44	3.87 ± 0.55
24	29	1.54 ± 0.15	2.07 ± 0.20	2.08 ± 0.17	0.14 ± 0.09	−0.03 ± 0.06	−0.28 ± 0.07	−0.17 ± 0.06	3.62 ± 0.30	5.69 ± 0.34
48	12	1.48 ± 0.12	2.48 ± 0.32	1.90 ± 0.14	0.17 ± 0.10	0.06 ± 0.09	−0.19 ± 0.09	0.11 ± 0.03	3.38 ± 0.23	5.87 ± 0.42
72	10	1.11 ± 0.12	2.09 ± 0.34	2.03 ± 0.23	0.21 ± 0.13	−0.05 ± 0.12	−0.25 ± 0.12	−0.27 ± 0.07	3.14 ± 0.29	5.22 ± 0.50
96	5	1.33 ± 0.25	2.69 ± 0.40	1.64 ± 0.26	0.32 ± 0.06	0.22 ± 0.11	−0.05 ± 0.08	−0.10 ± 0.09	2.97 ± 0.43	5.66 ± 0.66
144	3	2.20 ± 5.7	2.87 ± 0.12	2.31 ± 0.30	0.14 ± 0.09	0.10 ± 0.04	−0.18 ± 0.06	−0.04 ± 0.08	4.51 ± 0.82	7.38 ± 0.92
192	3	3.87 ± 1.0	3.84 ± 0.32	2.48 ± 0.54	0.02 ± 0.08	0.21 ± 0.09	−0.21 ± 0.03	0.18 ± 0.16	6.35 ± 0.94	10.2 ± 1.3

TABLE 4. MEAN DIFFERENCES IN PHEROMONE QUANTITIES AND LOG_{10} RATIOS FROM PAIRED COMPARISONS AGE TEST

Age 2–Age 1 (hr)	N	Amount (μg)			Ratio				C27 + C29 (μg)	Total (μg)
		C27	13MH	C29	13MH/C27	13MH/C29	13MH/(C27 + C29)	C27/C29		
3–1	12	0.00	0.05	−0.13	0.22	0.39	0.31	0.17	0.09	0.14
6–3	36	0.19	0.38[a]	0.48[b]	0.18[a]	0.02	0.09	−0.15[a]	0.62[b]	1.00[b]
9–6	9	1.09	2.44	1.07	0.27[a]	0.13	0.21[a]	−0.14	2.35	4.79
12–9	11	−0.18	0.09	−0.33	0.02	−0.01	0.01	−0.03	−0.28	−0.19
16–12	15	−0.04	−0.08	0.12	−0.02	0.00	0.01	0.02	−0.17	−0.25
24–16	12	−0.38	0.10	0.31	0.08	0.08	0.11	−0.01	−0.38	−0.07
48–24	7	0.38	0.78	0.83[a]	−0.01	0.01	0.00	0.02	0.90	1.67
72–24	10	−0.30	−0.53	0.61[a]	−0.17	−0.16	−0.14	0.02	−0.46	−0.98
96–48	5	−0.19	−0.06	0.13	0.07	0.07	0.06	0.00	−0.43	−0.49
192–144	3	1.67	0.97	0.28	−0.12[c]	0.10	−0.03	0.22	1.84	2.81

[a] Significant at 0.05 level.
[b] Significant at 0.01 level.

TABLE 5. PHENOTYPIC CORRELATIONS BETWEEN PHEROMONE COMPONENT QUANTITIES AT DIFFERENT AGES

Age (hr)	*N*	C27 vs. 13MH	C29 vs. 13MH	C27 vs. C29
1	12	0.43	0.21	0.56
3	22	0.04	0.04	0.11
6	19	0.49[a]	0.73[b]	0.26
9	20	0.98[c]	0.99[c]	0.97[c]
12	26	0.39[a]	0.48[a]	0.50[b]
16	26	0.61[d]	0.57[b]	0.47[a]
24	29	−0.28	0.13	0.64[d]
48	12	0.32	0.01	0.54
72	10	0.19	0.23	0.27
96	5	0.68	−0.18	0.41
144	3	0.77	0.99[a]	0.73

[a] Significant correlation at 0.05 level.
[b] Significant correlation at 0.01 level.
[c] Significant correlation at 0.0001 level.
[d] Significant correlation at 0.001 level.

TABLE 6. COMPARISON OF PHEROMONES AMONG FRESH (F) AND WORN (W) MALES, LAWRENCE, KANSAS (MEANS)

		1986		1987	
		F(36)	W(9)	F(25)	W(30)
μg:	C27	1.05	2.36[a]	1.14	2.00[a]
	13MH	6.35	4.30[b]	4.52	2.87[a]
	C29	2.48	2.56	2.72	2.80
	(C27 + C29)	3.53	4.92[c]	3.86	4.80[b]
	Total	9.88	9.22	8.38	7.67
Log_{10}:	$\frac{13MH}{C27}$	0.77	0.28[a]	0.55	0.14[a]
	$\frac{13MH}{C29}$	0.40	0.24[b]	0.17	−0.04[c]
	$\frac{13MH}{(C27 + C29)}$	0.24	0.07[a]	0.01	−0.29[a]
	$\frac{C27}{C29}$	−0.37	−0.04[c]	−0.38	−0.18[a]

[a] Significant difference at 0.001 level.
[b] Significant difference at 0.05 level.
[c] Significant difference at 0.01 level.

TABLE 7. PHENOTYPIC CORRELATIONS AMONG PHEROMONE COMPONENT QUANTITIES FOR FRESH AND WORN MALES, LAWRENCE, KANSAS

Year	Class	C27 vs. 13MH	C29 vs. 13MH	C27 vs. C29
1986	Fresh	0.30	0.41[a]	0.52[b]
	Worn	−0.27	0.00	0.09
1987	Fresh	0.47[a]	0.35	0.56[b]
	Worn	−0.13	−0.01	0.58[b]

[a] Significant correlation at 0.05 level.
[b] Significant correlation at 0.01 level.

TABLE 8. MEAN DIFFERENCES IN PHEROMONE QUANTITIES AND LOG_{10} RATIOS FROM PAIRED-COMPARISONS VOLATILIZATION EXPERIMENTS (FRESH–EXPOSED)

		3 hr @ 38°C (N = 18)	24 hr @ 54°C (N = 16)
μg:	C27	0.013	0.299
	C29	0.063	0.246
	13MH	0.097	0.032
	(C27 + C29)	0.202	0.838[a]
	Total	0.299	0.870[b]
Log_{10}:	$\frac{13MH}{C27}$	0.029	0.004
	$\frac{13MH}{C29}$	−0.032	−0.092
	$\frac{13MH}{(C27 + C29)}$	−0.004	−0.043
	$\frac{C27}{C29}$	−0.061	−0.096

[a] Significant difference at 0.01 level.
[b] Significant difference at 0.05 level.

DISCUSSION

Male pierids typically disseminate courtship pheromones from specialized scent scales on the wings (Bergstrom and Lundgren, 1973; Rutowski, 1977, 1980). Little is known about the process of secretion onto the wing scales in most

TABLE 9. COMPARISON OF PHEROMONES AMONG COURTING AND FEEDING MALES, LAWRENCE, KANSAS 1987 (MEANS ± SE)

		Courting (N = 41)	Feeding (N = 39)
μg:	C27	1.40 ± 0.14	1.48 ± 0.19
	13MH	4.80 ± 0.39	4.69 ± 0.39
	C29	3.30 ± 0.22	3.29 ± 0.22
	(C27 + C29)	4.70 ± 0.30	4.77 ± 0.37
	Total	9.50 ± 0.55	9.47 ± 0.57
Log_{10}:	$\frac{13MH}{C27}$	0.56 ± 0.05	0.53 ± 0.05
	$\frac{13MH}{C29}$	0.16 ± 0.03	0.11 ± 0.05
	$\frac{13MH}{(C27 + C29)}$	0.00 ± 0.04	−0.05 ± 0.05
	$\frac{C27}{C29}$	−0.41 ± 0.04	−0.42 ± 0.05

species, including *C. eurytheme*. The scent-producing scales of the closely related species *Colias philodice* are associated with individual cells in the integument (Rutowski, 1980), which are presumably secretory in nature, and the same situation may exist in *C. eurytheme*. Unlike the esters that make up the pheromone in the former species (Grula et al., 1980), however, the three hydrocarbons making up the *C. eurytheme* pheromone are typical cuticular hydrocarbons found in many insects. In fact, C27 and C29 are found on the wings of female *C. eurytheme* in quantities comparable to that of males (Grula et al., 1980; Sappington, 1989; Sappington and Taylor, 1990a). In the whitemarked tussock moth (*Orgyia lencostigma*), several odd-numbered *n*-alkanes on the scales, including C27, release male copulatory behavior after it has been stimulated with the female's long-range pheromone (Grant et al., 1987). The authors proposed that these common hydrocarbons act as sign stimuli to elicit copulation behavior after the male has made contact with the pheromone source. Evidence from the house fly, *Musca domestica* (Howard and Blomquist, 1982) suggests that it does not produce its cuticular pheromone in unique cells or glands, but rather in the same (perhaps modified) cells as other cuticular hydrocarbons, and this might be true for *C. eurytheme* also. Cuticular hydrocarbons in general are synthesized in cells associated with the epidermis or the peripheral fat body (Howard and Blomquist, 1982). Transport to the surface is prob-

ably through pore canals that penetrate the endocuticle and epicuticle (Jackson and Baker, 1970).

In the fly *Sarcophaga bullata*, there is a period of moderate hydrocarbon accumulation (5–8 μg/day) during the four days preceding ecdysis, and synthesis is controlled by ecdysterone (Arnold and Regnier, 1975). There is evidence that production of cuticular pheromones in the house fly *Musca domestica* (Howard and Blomquist, 1982) and the bruchid beetle *Zabrotes subfasciatus* (Pimbert and Pouzat, 1988) is induced by factors released at the onset of vitellogenesis. In *Pieris melete*, the total quantity of four pheromone components increases for the first two days and remains relatively constant thereafter through 100 hr/of age. During this period each of the components increases in quantity at different rates, causing changes in blend composition (Kuwahara, 1979). The author also concluded that the total quantity changed with time of day (1200 hr vs. 1800 hr), but this is based on a sample of only four and three individuals per group, respectively, whose ranges overlap, certainly making this an unwarranted inference. Lundgren and Bergstrom (1975) reported that pheromone production and deposition onto the scent scales of the lycaenid butterfly, *Lycaeides argyrognomon*, started during the first day posteclosion in lab-reared stock, but no details were given.

In *Colias eurytheme* the greatest accumulation of all three pheromone components occurs between 3 and 9 hr posteclosion (Table 4). It also is apparent from the ratio data (Table 4) that during the first 12 hr, 13MH is deposited more rapidly than C27 and C29, and C27 more rapidly than C29. Blends remain remarkably constant throughout life after the first 12 hr, even though quantities of the components fluctuate a bit, suggesting that any small age-related changes after this period affect all components equally.

In *Colias philodice*, the ability of male wings to induce an acceptance response in females was reduced significantly by exposure of detached wings to room temperature for 24 hr, suggesting significant volatilization of one or more pheromone components (Rutowski, 1980). There is no evidence that any of the components in the *C. eurytheme* pheromone volatilize to any appreciable degree at temperatures that are experienced in the field (Table 8) or even elevated temperatures above those reached while basking or overheating (Watt, 1968, Watt et al., 1983; Kingsolver and Watt, 1983; Kingsolver, 1983a, b). Nor does the greater flight activity of actively searching males cause a detectable loss of pheromone (Table 8). Watt et al. (1985) have shown that males with PGI genotypes that permit more flight activity under prevailing climatic conditions are more successful at obtaining copulations than those with less kinetically favorable genotypes. They propose that this differential success is related to differences in male persistence during courtship. It is not known if PGI genotype has pleiotropic effects on pheromone composition, but the lack of differences in the pheromones of courting vs. resting males (Table 9) indi-

cates that PGI genotype is not correlated with pheromone phenotype through the effects of differential volatilization associated with differential flight activity.

The low volatility of the components (Table 8) ensures that depletion will occur very slowly over the life of the male. Low volatility also requires that this pheromone be detected by females either via contact or aerially at very close range. In bioassays in which detached wings were presented to females, contact of the wings with the antennae was necessary to elicit a response in *Eurema lisa* (Rutowski, 1977), *Colias philodice* (Grula et al., 1980; Rutowski, 1980), and *C. eurytheme* (Grula et al., 1980; Sappington, personal observation). In all of these species, courting males flutter vigorously around the perched female and buffet her with their wings (Rutowski, 1980; Grula et al., 1980).

Pheromone composition in *C. eurytheme* does seem to change with increased wing wear in wild populations, although not in a way that is readily interpretable (Table 6). The quantity of 13MH is less in worn males than in young ones, which is to be expected if 13MH is carried on scales that are lost as the butterflies age. However, the quantity of C27 increases with wing wear, suggesting that it continues to be secreted. The data from the age tests (Table 2–4), however, indicate C27 does not increase with age, at least between the ages of 12 hr and 96 hr. The situation after 96 hr posteclosion is less clear. The noctuid moth, *Trichoplusia ni*, emerges from the pupa with the wax layer consisting primarily of methylalkanes that are produced only in the larval stage, but *n*-alkanes, produced exclusively by adults, increase in amount to day 5 (De Renobales and Blomquist, 1983). It is possible that C27 is produced by older *C. eurytheme* males to replace the molecules lost during courtship or through wing wear, while 13MH is not. Elucidation of this problem awaits further study.

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Book Review

Chemical Mediation of Coevolution. Kevin C. Spencer (ed.). San Diego, CA: Academic Press, 1988. 609 pp. illustrated. $95.00; $39.95 (paper).

Kevin Spencer's edited volume has become available, appropriately, at essentially the 25-year anniversary of Ehrlich and Raven's classic paper "Butterflies and plants: A study in coevolution." (Evolution 18:584–608, 1964). The term coevolution was coined in this paper, and it subsequently became highly popularized to describe any aspect of plant–organism interactions, but particularly those between plants and insects. Ehrlich and Raven, however, emphasized that the process by which plants and insects radiate and speciate in association with one another involves the evolution of chemical defenses by plants against insect herbivores.

In this volume Spencer has drawn together some prominent plant–herbivore researchers to "specifically address chemistry as a factor in establishment and maintenance of coevolution and to test coevolutionary concepts for their pertinence to chemically mediated systems" (p. 9). He, as have others such as Futuyama and Slatkin, points out that previous literature has concentrated on adaptations resulting from coevolution rather than a mechanistic interpretation of the process. Therefore, he hypothesizes that some aspects of chemistry should provide a *mechanistic* explanation for diversification of the interacting systems of plants and insects. Despite Spencer's obviously enthusiastic high hopes, the book only succeeds in presenting an interestingly diverse array of current research and viewpoints regarding chemical mediation of coevolution.

A major problem that has plagued coevolutionary studies since Ehrlich and Raven's coinage of the term is the lack of a commonly accepted definition of coevolution. Some scientists refer to the process so broadly that it becomes equivalent to evolution, whereas others require genetic reciprocity as part of their definition. The pairwise reciprocal coevolutionary model predicts a "chemical arms race," but here Gould in his excellent genetic analysis of the process suggests that gene-for-gene reciprocity probably is not common. Rather, he thinks that process generally involves more than two species or foci, and hence the necessity to qualify the process as "diffuse or multispecies coevolution."

Although many of the researchers in the 16 chapters of this book obviously had difficulty in ascertaining that their systems were coevolved, some papers

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present research that somewhat parallels Ehrlich and Raven's scenario. Spencer, working with the classic system of *Passiflora* and *Heliconius*, has made extensive analyses of the cyanogenic glycosides and provides a hypothesis as to why their toxic effects may vary and how *Heliconius* species may differ in avoiding toxicity. His statistical correlations of host use with the chemistry, however, are difficult to understand, as is his conclusion that *Heliconius* and *Passiflora* have coevolved in the reciprocal stepwise manner. Also, Bowers discusses plants containing iridoid glycosides, which are important at several levels to adapted specialist insects, as an exemplary system for study of short-term reciprocal coevolutionary changes.

Berenbaum's previous studies of the association of insect herbivores and host plants in the Umbelliferae have shown the furanocoumarins to correspond to insect specialization in ways that fit the Ehrlich and Raven scenario, but she and Zangerl present some of the "stalemates in the coevolutionary arms race" in their chapter. They emphasize that insects are not the sole selective force maintaining furanocoumarin variation in parsnips and that different agents of plant stress select for different combinations of chemicals. They further point out that "particular chemical fitness traits in a plant may be adaptive only under a particular set of environmental conditions" (p. 128).

Some authors, although not questioning that chemicals play a role in plant–animal interactions, are skeptical that chemicals are significant to the process of reciprocal coevolution. For example, Chew summarizes extensive studies of crucifer–glucosinolate–herbivore interactions in which she demonstrates that chemistry in some cases can be important. However, she concludes that it is not mediating the specificity of these interactions and, hence, coevolution in this system. In a different approach, Craig et al. discuss forces preventing coevolution in a three-trophic-level system, but nonetheless show that chemistry is involved in interactions at each level. In so doing, they suggest that in elucidating the mechanisms of coevolution there is value in studying systems where evidence for coevolution is present and systems where it is absent. As an alternative to coevolution of cardinolide-based chemical defense, Brower et al. invoke exaptation—both in terms of defensive sequestration of the chemical by monarch butterflies and circumvention of this defense by orioles.

In the only chapter on plant and mammal interactions, Lindroth points out that they show "chemically oriented adaptations," (p. 437) but suggests that there is little evidence for chemical coevolution because of the diffuse nature of the selective forces occurring between these generalist herbivores and plants. Myers also thinks that plant defense fits into a more diffuse rather than a tightly coevolved system and doubts the validity of the induced chemical defense hypothesis in determining population dynamics of insects. She agrees with those who express the need for a "more holistic view of selection pressures acting on plants" (p. 363) to understand their responses to damage. In a detailed study

of *Papilio* biogeography, Scriber turns to factors other than chemistry determining the insects' food choice. He further reasons that it is unlikely that *Papilio* have either shaped the chemistry or fitness of the various host trees, which lack a "chemical common denominator."

Other authors present noteworthy chemical research that seems to address coevolution somewhat tangentially if at all. For example, we must assume that Cates and Redak's fine study of the defensive role of terpene variation in Douglas fir against spruce budworm success is placed in the coevolutionary context through a refinement of ideas in previous antiherbivore theory expressed by Cates (with Rhoades). In this and other theories that build upon Ehrlich and Raven's premises, coevolutionary interactions between plants and herbivores have been invoked to explain the types and quantities of resources allocated to chemical defense. However, Bryant et al. emphasize that abiotic environmental constraints of both constitutive and long-term inducible defenses must be determined before coevolutionary interactions between plants and herbivores can be presumed to be the primary cause of allocation of resources to plant chemical defense. In an entirely different kind of study, Stowe provides a plethora of fascinating information on chemical mimicry that derives ultimately from herbivory, but he does not specifically discuss coevolution.

This volume obviously is not an overview of coevolution or even the chemical mediation of it. An ecological perspective also is emphasized, with less attention given to the broader phylogenetic aspects. Although plant–insect interactions have been a particularly active area of recent research, at least one chapter could have discussed plant–microbe interactions where the gene-for-gene concept arose. The essays clearly demonstrate the confusion that still exists regarding the very concept of coevolution and the complexity of analyzing chemical mediation of plant–animal interactions—let alone being able to detect the mechanisms controlling a tightly evolved reciprocal system. However, Spencer's volume provides a compact, useful reference of excellent varied research with primarily cautious views regarding coevolution (however it may be defined!). Furthermore, these view are often accompanied by suggestions to encourage those researchers who undoubtedly will continue the quest to understand the origins and evolution of defensive chemicals as well as the possible role they may have played in the diversification of interacting organisms. Additionally, this collection of essays represents a milestone contribution in demonstrating how much stimulation of thought and effort Ehrlich and Raven have provoked during the last quarter of a century.

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ATTRACTION OF PINYON PINE BARK BEETLE, *Ips hoppingi*, TO CONSPECIFIC AND *I. confusus* PHEROMONES (COLEOPTERA: SCOLYTIDAE)

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Abstract—Females of a pinyon pine bark beetle, *Ips hoppingi* Lanier, were less attracted by the aggregation pheromone produced by conspecific males than by the pheromone produced by the neighboring sibling species, *I. confusus* (LeConte). Cross-attraction was elicited by males infesting the regional pinyon pine hosts (*P. discolor* and *P. edulis*) of either *Ips* species in southeastern Arizona. Pheromonal specificity has not accompanied speciation in this species pair.

Key Words—Pheromone, pinyon pine, bark beetle, cross-attraction, *Ips hoppingi*, *Ips confusus*, Coleoptera, Scolytidae, *Pinus discolor*, *Pinus edulis*.

INTRODUCTION

Bark beetles of the genus *Ips* communicate their colonization of the phloem of conifers using an aggregation pheromone incorporated into the male's frass. The pheromones of analyzed Nearctic, pine-infesting species of *Ips* typically contain one or more monoterpene alcohols. For example, ipsdienol, ipsenol, and *cis*-verbenol were identified from frass produced by recently emerged male *I. paraconfusus* and have been shown to be biosynthesized from host monoterpenes (reviewed by D.L. Wood, 1982; Borden, 1984, 1985). Pheromones of

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sympatric *Ips* species are not usually cross-attractive (Lanier and Wood, 1975). Sympatric *Ips* species often belong to different species groups. Conversely, the ranges of species belonging to the same species group rarely overlap (S.L. Wood, 1982). Pheromonal cross-attraction has been reported among these more closely related species (Lanier and Wood, 1975; Cane et al., 1990).

Two closely related species of *Ips* that are only known to infest pinyon pines (*Pinus* subsection *Cembroides*) have been described from western North America and Mexico. *Ips confusus* occurs throughout much of the desert southwestern United States. It has been collected from *Pinus monophylla* Torrey and Fremont, *P. edulis* Engelmann, *P. quadrifolia* Parlatore, and *P. californiarum* Bailey. To the south and east of the range of *I. confusus*, *I. hoppingi* attacks Mexican pinyon, *P. cembroides* Zuccarini, in Mexico and western Texas and border pinyon, *P. discolor* (Little) Bailey and Hawksworth, in the mountains of southern Arizona and New Mexico. (Lanier, 1970) (Table 1). It likely also occurs in Texas pinyon, *P. remota* (Little) Bailey and Hawksworth, in western Texas. The ranges of several of the respective pinyon hosts of these two beetle species are distributed contiguously in Arizona and New Mexico (Bailey and Hawksworth, 1979; D.K. Bailey, personal communication). For instance, we have seen *P. discolor* and *P. edulis* growing together at Mule Pass east of Safford, New Mexico, an observation confirmed by D.K. Bailey (personal communication). Only *I. hoppingi* was reported from pinyons in the Chiricahua Mountains of southeastern Arizona by Lanier (1970).

These two *Ips* species are inseparable by any reported macromorphological character. The density of ridges of the female's supracephalic pars stridens, a structure used in courtship communication, provides a reliable diagnostic character for distinguishing females of these two species (Lanier, 1970). This spe-

TABLE 1. LOCALITIES AND PINE HOSTS OF *I. confusus* AND *I. hoppingi* COLLECTED BY AUTHORS[a]

Ips species	*Pinus* Host	Locality
confusus	*monophylla*	e.g., 12 km NE Big Bear Lake, California (San Bernardino Mountains)
	edulis	e.g., Mimbres Ranger Station, New Mexico (Mimbres Mountains)
	quadrifolia	e.g., 15 km SE Idyllwild, California (San Jacinto Mountains)
	californiarum	13 km N Pioneer Town, near Twenty-nine Palms, California
hoppingi	*cembroides*	McDonald Observatory, near Ft. Davis, Texas (Davis Mountains)
	discolor	12 km W Portal, Arizona (Chiricahua Mountains)

[a]Taxonomic identities confirmed by authors, M.W. Stock, and S.J. Gast.

cies pair is also markedly similar electrophoretically (Nei's genetic identity = 0.929), but individuals can be readily grouped by species, as these two species are monomorphically fixed for a different allele at the *esterase* locus ($N = 360$ individual beetles, both sexes, from five populations). This difference is unaffected by host ($N = 6$ pinyon species), at least for populations we sampled from across their range (California, Arizona, New Mexico, Texas) in the American Southwest (Cane et al., unpublished). Crosses between these two species by Lanier (1970) yielded only inviable eggs, using *I. hoppingi* from the Chiricahua Mountains of Arizona and *I. confusus* from New Mexico. The close morphological, electrophoretic, and biological similarity of *I. confusus* and *I. hoppingi* suggests their recent phylogenetic divergence and potential for pheromonal cross-attraction.

In the only pheromonal study with *I. hoppingi*, females were strongly attracted to male *I. paraconfusus* Lanier aggregation pheromone in a laboratory olfactometer. To a lesser degree, female *I. paraconfusus* in these experiments were reciprocally attracted by the frass of *I. hoppingi* (Lanier and Wood, 1975). Our field experiments challenged male and female *I. hoppingi* to discern their host's volatiles and conspecific pheromone from those of *I. confusus* and one of its hosts, *P. edulis*. These two *Ips* species have been collected from populations as close as ca. 150 km from each other in the American Southwest (Lanier, 1970). Our objective was to assess the evolutionary divergence of their semiochemical communication systems. These are the first reported field tests of semiochemical responses by *I. hoppingi*.

METHODS AND MATERIALS

Logs were cut from living trees near Glenwood, New Mexico (*P. edulis*), and the Chiricahua Mountains in southeastern Arizona (*P. discolor*). The logs were air-dried for two to three weeks and then sawed into bolts presenting 1500 cm^2 of bark surface.

Male *I. hoppingi* were emerged from infested *P. discolor* found near our test site in the Chiricahua Mountains, along the road leading through Onion Saddle from the eastern side of the mountains. Adult *Ips* reared from infested *P. discolor* collected at this site in September 1985 were all *I. hoppingi* by electrophoretic analysis (Cane et al., unpublished). Male *I. confusus* were emerged from infested *P. edulis* cut near Mimbres, New Mexico, 150 km NE of our site of experimentation. Their identity was confirmed morphologically and by their lack of hybridization with *I. hoppingi* from our test site.

Twenty-five conspecific males were introduced to the phloem of a bolt via individual subcortical nail holes driven to the surface of the sapwood. Each of the four combinations of pinyon and *Ips* species was represented once (e.g., *I. confusus* infesting *P. discolor*, *I. confusus* infesting *P. edulis*, etc.). Eight

remaining bolts were left uninfested. All bolts were wrapped in aluminum screen to prevent volunteer attacks.

Bolts were distributed among four plots 100 m apart along the road leading through Onion Saddle on the eastern side of the Chiricahua Mountains, at the location of the 1985 electrophoretic sample. Each bolt was surrounded by a hardware cloth sticky trap and set atop a 1.5-m iron pipe standard. An uninfested bolt of each of the two pinyon species was placed 1 m to each side of the single infested bolt in each plot. In five similar experiments over three years with three group IX *Ips* species, this distance was found to be close enough to attract responding males into the vicinity of uninfested bolts, yet distant enough to generate consistently different sex ratios among *Ips* responding to uninfested and infested bolts (Cane et al., 1990). Beginning on August 26, 1987, all sticky traps were checked and then randomized daily among plots after removal of trapped beetles (methods detailed in Cane et al., 1990).

We statistically assessed three hypotheses: (1) males of *I. hoppingi* preferentially arrive at uninfested bolts of host rather than nonhost pinyon; (2) joining males (those captured at the male-infested bait bolts) preferentially land at host rather than nonhost bolts, and (3) joining female *I. hoppingi* preferentially land at host *P. discolor* infested by conspecific males when compared to any of the three other combinations of pinyon and infesting beetle species. Hypothesis 1 and 2 were tested by a Wilcoxon two-sample test, paired within days. For responding females, we chose Friedman's method for randomized blocks (blocked by days), with the four bolt/beetle combinations as treatment effects (Sokal and Rohlf, 1981). Rejection of the null hypotheses was set at $P < 0.05$.

Taxonomic identities of a sample of 75% of the responding females were assigned using the densities of striae of their cephalic pars stridens. Measurements were taken from scanning electron microscope images at 10,000–16,000× after internal calibration using latex spheres (Ted Pella Inc., Redding, California) (methods after Cane et al., 1990). Measurements of trapped females were compared to those from populations of: (1) *I. confusus* collected from naturally infested *P. edulis* in Grant County, New Mexico, and presumed *I. hoppingi* from *P. discolor* baited with *I. confusus* pheromone in the Chiricahua Mountains of southeast Arizona, near our test site.

Clerid beetles attracted to the traps were identified by William F. Barr, University of Idaho. Voucher beetles are deposited with the Essig Museum (University of California, Berkeley) and the Auburn University collections, Auburn, Alabama.

RESULTS

Experimentally introduced and confined males of both *Ips* species readily excavated nuptial chambers in both pinyon species within 12 hr (no fewer than 23 of the 25 males/bolt), indicative of their acceptance of nonhost pinyon and

their general vigor. Over the six-day experiment, 161 *Ips* beetles were collected, 80% of them at the infested bolts. Using the pars stridens measures, we classified 95% of the responding females as *I. hoppingi*; three of the 73 females measured had striation widths more comparable to *I. confusus* (Figure 1). No other known *Ips* species, such as *I. lecontei*, was trapped. A significant proportion (70%) of female *I. hoppingi* collected at the four infested bolts were trapped at the two bolts infested by male *I. confusus* (N = 35 and 39 females) (Figure 2). Furthermore, 46% of the females were trapped at infested bolts of the nonhost, *P. edulis*. Twenty-six females (20% of the total catch) were trapped at the uninfested bolts.

At the uninfested and infested bolts, too few males were trapped for meaningful statistical comparisons (N = 8 and 21 males, respectively). Notably, 76% of the joining males were trapped at the infested host, *P. discolor*, rather than infested nonhost. The two infesting *Ips* species attracted equal numbers of *I. hoppingi* males (10 vs. 11 males).

Female *I. hoppingi* did not arrive in significantly greater numbers at the combination of host pine and conspecific pheromone (T = 4.45, one-tailed $P > 0.2$). If daily collections are pooled by the infesting beetle species, then daily catches of *I. hoppingi* females were always greater at bolts infested with *I. confusus* than conspecific males. A Wilcoxon signed-ranks test (Sokal and Rohlf, 1981) of these pooled data shows this difference to be significant ($P < 0.03$) (Figure 2).

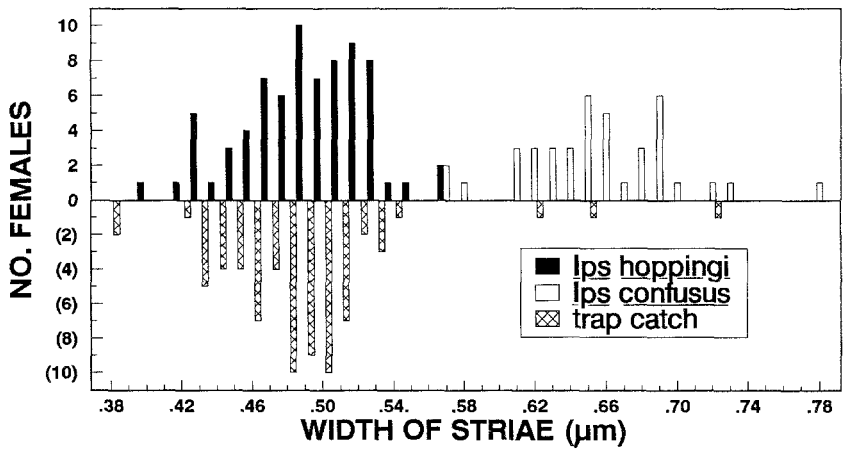


FIG. 1. Identities of responding female *Ips* trapped during this experiment. Measurements are the width of single striae from the cephalic pars stridens. Comparisons are with known populations of *I. hoppingi* (SE Arizona), and *I. confusus* (S New Mexico) collected from naturally infested material. Pinyon pine identifications conform to Bailey and Hawksworth (1979) and Bailey (1987).

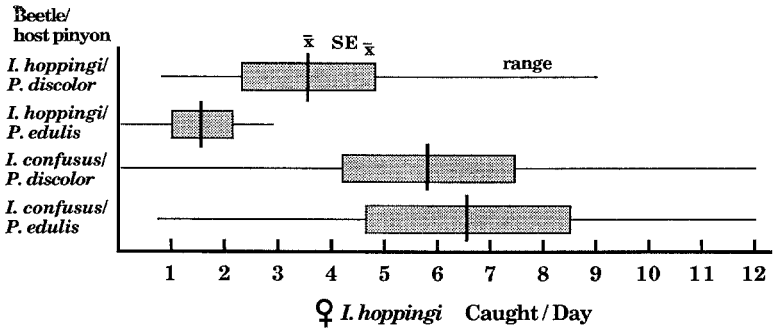


FIG. 2. Responses of female *I. hoppingi* to semiochemical combinations produced by conspecific or *I. confusus* males boring in bolts cut from the pinyon pines *P. discolor* or *P. edulis*. Significantly more females were attracted to the bolts infested by male *I. confusus* (pooled by host) than bolts infested by conspecific males. No one beetle–host combination was significantly more or less attractive than any other.

The clerid *Enoclerus lecontei* (Wolcott) was the most abundant predator trapped ($N = 80$ individuals). They were 3.2 times more likely to be trapped at an infested than an uninfested bolt. Two less commonly trapped clerids were *Phyllobaenus arizonicus* (Schaeffer) ($N = 14$) and *E. arachnodes* (Klug) ($N = 1$). Thirteen *Temnochila chlorodia* (Trogositidae) (Mannerheim) also were caught.

DISCUSSION

In this experiment, more female *I. hoppingi* were attracted to the aggregation pheromone released by male *I. confusus* than by conspecific male pheromone. They were attracted to males tunneling in both host and nonhost pinyon pines. While such extreme cross-attraction may reflect idiosyncrasies of the individual beetles used, female *I. hoppingi* clearly failed to respond preferentially to conspecific pheromone. Until the pheromone of *I. hoppingi* is identified and until the identified pheromone of *I. confusus* is tested in the field, we cannot explain the demonstrated cross-responsiveness of these two species in nature. Given the close morphological, biological, and electrophoretic similarity of these two species, it appears from our experiments that they are diverging from common ancestry without any accompanying functional divergence in their semiochemical communication systems.

These two *Ips* species likely have had prolonged opportunity for both interaction and invasion of each other's hosts. The post-Pleistocene distributions of their pinyon hosts are evidenced in the macrofossil plant assemblages from the middens of *Neotoma* pack rats (summarized by Lanner and Van Devender, 1981;

Betancourt, 1987; Wells, 1987). Although distributions of most pinyons have dramatically shifted, expanded, or contracted over the past 15,000 years, there have continued to be regions of sympatry between the present-day hosts of *I. hoppingi* and *I. confusus*. For example, middens dated at 14,060 + 390 BP from the Santa Catalina Mountains of southern Arizona contain macrofossils of *P. discolor*, *P. edulis*, and *P. monophylla* (Thompson and Van Devender, 1982). Our pars stridens measurements of responding females suggest that these two *Ips* species may be narrowly sympatric in the Chiricahuas today (Figure 1).

The contiguous distribution of these *I. hoppingi* and *I. confusus* exemplifies Jordon's (1905) observation that closely related species tend to be geographically isolated. Lanier and Wood (1975) and Cane et al. (1990) noted this phenomenon for a number of North American *Ips* species. They suggested that the lack of pheromonal specificity among closely related *Ips* species may often enforce their disjunct distributions, owing to sterile interspecific matings. Our field experiments with *I. hoppingi* and *I. confusus* support their contention, but leave open the possibility of host and/or mate specificity following bark contact by the responding beetles.

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We dedicate this paper to the memory of the late Professor Gerald N. Lanier, who discovered and described I. hoppingi. *His multidisciplinary approach to the biosystematics of the genus Ips was a pioneering contribution that will form the foundation for research on other taxa in the Scolytidae.*

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A NOVEL ATTRACTANT FOR MEXICAN FRUIT FLY, *Anastrepha ludens*,[1] FROM FERMENTED HOST FRUIT

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Abstract—Chemicals from fermented chapote fruit were identified and evaluated as attractants for hungry adult Mexican fruit flies in laboratory and greenhouse bioassays. Twenty-eight chemicals identified from an attractive gas-chromatography fraction were as attractive as a chapote volatiles extract (CV) when mixed in the same amounts found in CV. Sixteen of the chemicals were slightly attractive to flies when tested individually. A mixture containing 15 of the chemicals by design and the 16th as an impurity, in arbitrary concentrations, was at least as attractive as the original CV. In a series of experiments, the number of chemicals was reduced to three by elimination of unnecessary components. The three-component mixture retained the attractiveness of the 15-component mixture. The three chemicals were 1,8-cineole, ethyl hexanoate, and hexanol (CEH). Attractiveness of the three-chemical mixture was equal to the sum of the attractiveness of the three individual components, suggesting that each chemical binds to a different receptor type that independently elicits partial attraction behavior. Optimal ratios were 10:1:1 of the three chemicals, respectively. Optimal test quantities ranged between 0.4–4 μg of 1,8-cineole and 40–400 ng each of ethyl hexanoate and hexanol applied to filter paper in the laboratory bioassays. A neat 10:1:1 mixture of the chemicals was 1.8 times more attractive than aqueous solutions of *Torula* dried yeast and borax to starved 2-day-old flies when the lures were tested in competing McPhail traps in a large greenhouse cage.

Key Words—Attractants, Mexican fruit fly, Diptera, Tephritidae, *Anastrepha ludens*, host fruit, yellow chapote, Rutaceae, *Sargentia greggii*.

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INTRODUCTION

Fermenting sugars, yeasts, and protein hydrolysates combined with water in McPhail traps (Shaw et al., 1970) have been the standard lures for various species of *Anastrepha* fruit flies for over 50 years. Laboratory and field screening of thousands of candidate attractant chemicals during 1955–1963 by J.F. Cooper, D.L. Chambers, F. Lopez-D, and O. Hernandez-B (Shaw et al., 1970), over a thousand during 1972–1978 by Burditt and McGovern (1979), and thousands again during 1982–1988 by W.G. Hart (personal communication), discovered no promising attractants for *Anastrepha*. Further, Lopez-D and Chambers evaluated extracts of 456 plant species and also found no promising attractants (Shaw et al., 1970). Baker et al. (1944) reported that Mexican fruit fly (*Anastrepha ludens* Loew) adults frequently were seen feeding on fallen mangoes, suggesting that the fermenting fruit was attractive to the flies. However, no attractant was developed from this or any fruit for *Anastrepha* species. Finally, Hedström (1988) demonstrated that human urine was 10 times more attractive to *A. striata* and *A. obliqua* than aqueous solutions of *Torula* dried yeast and sodium borate in McPhail traps, but no attractive chemicals have been identified.

Robacker et al. (1990) demonstrated that the odor of fermented chapote fruit, a native host of *A. ludens*, was highly attractive to hungry adult *A. ludens*. In greenhouse experiments, fermented chapote fruit was 3.6 times more attractive than aqueous solutions of *Torula* dried yeast and borax (YB) at distances of <0.3 m. These promising results led to the current work to develop from chapote odor a mixture of chemicals attractive to hungry *A. ludens*.

Specifically, objectives of this research were to: identify chemicals in fermented chapote odor attractive to *A. ludens*; prepare a mixture of the chemicals with attractiveness equal to chapote odor; eliminate unnecessary chemicals; determine optimal component ratios and concentrations; and evaluate the final mixture against YB, one of the most commonly used lures for *A. ludens*, in greenhouse tests.

METHODS AND MATERIALS

Insects and Test Conditions. Flies were from a culture maintained for at least 70 generations with no wild-fly introductions. Pupae were irradiated with 7000–9200 rads (cobalt-60 source) one to three days before adult eclosion in the YB/CEH comparison experiment because it was conducted in a greenhouse. Flies used in other experiments were not irradiated. Flies were tested when 2-3 days old: 2-day-old flies were never fed as adults; 3-day-old flies were fed sugar until one day before tests to prevent excessive mortality. Flies were not deprived

of water. Mixed-sex groups of 140–200 flies were used. Unless stated otherwise, tests were conducted between 0900 and 1400 hr in the laboratory under a combination of fluorescent and natural light. Responses to chapote odor are relatively constant during this period of the day (Robacker et al., 1990). Laboratory conditions were 22 ± 2°C, 50 ± 20% relative humidity, and photophase from 0600 to 2000 hr.

Chapote Volatiles (CV). The fermented, immature chapote fruit was described previously (Robacker et al., 1990). Between 70 and 150 g of the fruit (ca. 150–300 fruits) were put into a cylindrical glass aeration chamber (26 cm long × 8 cm ID) with an airflow of about 100–120 ml/min. The chamber was fitted with both an air filter and a trapping tube each containing Porapak Q (Waters Associates, Milford, Massachusetts) (50/80 mesh). The Porapak Q was precleaned with successive rinses of acetone, dichloromethane, and 10% dichloromethane in pentane. Volatiles were extracted with 10% dichloromethane in pentane following 24 hr of fruit aeration. The extract was stored at −20°C.

Gas Chromatography–Mass Spectrometry. Chemicals in CV were identified by gas chromatography–mass spectrometry (GC-MS). A Finnigan MAT 4500 GC-MS data system (Finnigan MAT, San Jose, California) was operated in the electron-impact ionization mode (emission current = −0.30 mA, electron energy = 70 eV, multiplier voltage = −1225 V). Mass spectral data were acquired over a mass range of 33–350 amu, using a 1-sec scan rate. Several columns were employed: DB-1 and DB-Wax (J & W Scientific, Rancho Cordova, California) (60 m, fused silica, 0.32 mm ID, 0.25 and 1.0 μm film).

Gas Chromatography (GC). We used a Shimadzu GC-9A instrument (Shimadzu Scientific Instruments, Inc., Columbia, Maryland) with a flame ionization detector. Analyses were on either a DB-225 Megabore column (J & W Scientific) (30 m, fused silica, 0.53 mm ID, 1 μm film) or a SPB-1 capillary column (Supelco, Inc., Bellefonte, Pennsylvania) (60 m, fused silica, 0.32 mm ID, 1 μm film).

Chemicals Evaluated in Bioassays. Twenty-eight chemicals identified in CV by GC-MS were obtained for behavioral assays. Chemicals, sources, and purities are listed in Table 1. Purities stated on product labels were verified by GC. Note that Table 1 lists specific enantiomers for several chemicals even though GC-MS does not distinguish optical isomers. In most cases, enantiomers were chosen because they are the predominant isomers found in natural plant sources.

Chapote Volatile Synthetic Mixture (CVSM). The 28 chemicals listed in Table 1 were mixed together approximately in the same amounts as in CV. Table 1 shows the concentrations of the chemicals in CVSM. CVSM was used as a standard in attractiveness tests of various mixtures of chemicals.

Tandem GC–Behavior Bioassay. This bioassay was used to evaluate attractiveness of chemicals as they eluted from the GC column: DB-225 Megabore;

TABLE 1. CHEMICAL NAMES, SOURCES, PURITIES, AND CONCENTRATIONS IN CVSM TEST MIXTURE OF 28 CHEMICALS EVALUATED BEHAVIORALLY IN VARIOUS EXPERIMENTS

Chemical	Source[a]	Purity[b] (%)	Amounts in CVSM (ng/μl)
Ethyl butyrate	Sigma	99	1
(+)-α-Pinene	Sigma	99	445
Ethyl 2-methylbutyrate	Aldrich	99	1
3-Methylbutanol	Sigma	99	36
(*S*)-(−)-2-Methylbutanol	Sigma	99	12
(+)-Camphene	Aldrich	80	4
Hexanal	Sigma	98	1
3-Methyl-3-buten-1-ol	Aldrich	97	2
Pentanol	Sigma	99	1
3-Methylbutyl acetate	Sigma	98	1
β-Pinene	PCR	95	12
(+)-Sabinene	Aldrich	99	4
3-Methyl-2-buten-1-ol	Aldrich	99	1
β-Myrcene	Sigma	90	76
α-Phellandrene	P&B	97	2
α-Terpinene	PCR	88	4
Methyl hexanoate	Alltech	99	1
D-Limonene	Sigma	97	117
β-Phellandrene	unknown	80	17
Hexanol	Sigma	99	1
γ-Terpinene	Sigma	95	107
cis-3-Hexen-1-ol	Sigma	97	2
1,2,4-Trimethylbenzene	Sigma	98	7
1,8-Cineole	Aldrich	99	<1
Ethyl hexanoate	Alltech	99	2
trans-2-Hexen-1-ol	Aldrich	97	1
Dodecane	Alltech	99	2
p-Cymene	PCR	95	95

[a] Sigma Chemical Company, St. Louis, Missouri; Aldrich Chemical Company, Inc., Milwaukee, Wisconsin; PCR Research Chemicals, Inc., Gainesville, Florida; Pfaltz & Bauer, Inc., Waterbury, Connecticut; Alltech Associates, Inc., Deerfield, Illinois.
[b] Determined from label information and GC analysis.

50°C for 5 min, 1°/min increase until 120°C isothermal. A splitter at the end of the column diverted ca. 95% of the effluent through a cage (20 cm/side) containing 200 2-day-old flies. All flies within a 5-cm-diam. circle around the effluent entry point were counted every 15 sec for 80 min. Twenty replications were conducted for CV and 22 for CVSM.

Cage-Top Bioassay. Cage-top bioassays were used to evaluate attractiveness of chemicals and mixtures of chemicals in most experiments (Robacker and Hart, 1984; Robacker et al., 1990). A response index (RI) was defined as the ratio of the number of flies attracted to treatments vs. solvent controls (Robacker et al., 1990).

Tests of Individual Chemicals. Solutions of the 28 chemicals in Table 1 were prepared in hexane and tested using cage-top bioassays. Two amounts of each chemical were tested: 400 ng in 10 μl and 4 μg in 100 μl (except 1,8-cineole). 1,8-Cineole was not identified in the initial GC-MS work. When it was identified later, we tested it at 40 ng in 10 μl and 400 ng in 100 μl to reflect its concentration in various mixtures in which it was present as a contaminant of α-terpinene. Further, it was tested during mixture experiment 8 instead of at the same time as the other individual chemicals. Ten replications each were conducted for low and high amounts.

Mixture Experiments. These experiments were conducted to determine a minimum set of chemicals as attractive as the original CV. Chemicals evaluated in the individual-chemical tests that were more attractive at the lower test quantity were incorporated into mixtures at 4 ng/μl and those more attractive at the higher quantity at 40 ng/μl. Two quantities of each mixture were tested, 10 μl and 100 μl, using cage-top bioassays. Ten replications each were conducted for both 10- and 100-μl amounts. The composition of the various mixtures is shown in Table 2. Mixture names reflect the number of chemicals they contained by design, although others were present as impurities. Eight mixture experiments were conducted (Table 3).

The first mixture experiment evaluated six mixtures. Mix 15 contained all chemicals (including 1,8-cineole as an impurity of α-terpinene) that outperformed the blank in the individual-chemical tests at either the low amount, the high amount, or over both amounts. Other mixtures in the first experiment contained fewer chemicals, each deletion based on lower attractiveness in the individual-chemical tests.

For experiments 2–4, each successive experiment was based on results of the preceding one. Generally, the minimum-chemical mixture from each experiment that retained attractiveness equal to CVSM was used as the maximum set of chemicals from which further eliminations were made.

Mixture experiment 5 was performed to evaluate further the attractiveness of *D*-limonene. Mix 3G contained hexanol and ethyl hexanoate at the same concentration as in mix 4D, and α-terpinene at 80 ng/μl, twice the concentration in mix 4D, to substitute for D-limonene.

Mixture experiment 6 was conducted to determine if the attractiveness attributed to the commercial-grade α-terpinene (Table 1: 88% purity) was due to α-terpinene or to contaminants. Commercial α-terpinene was purified by high-performance liquid chromatography (HPLC). A Waters HPLC (Waters Asso-

TABLE 2. CHEMICAL CONCENTRATIONS (ng/μl) IN MIXTURES USED IN MIXTURE EXPERIMENTS 1–8

	Mix												
Chemical	15	12	8	7A	7B	7C	6	5A	5B	4A	4B	4C	4D
Ethyl butyrate	40												
Ethyl 2-methyl-butyrate	40	40	40	40	40								
Pentanol	4	4	4	4		4							
(+)-Sabinene	40	40	40	40	40	40	40	40	40		40	40	
β-Myrcene	40												
α-Phellandrene	40	40											
α-Terpinene													
88%	40	40	40	40	40	40	40	40	40	40	40	40	40
99.7%													
Methyl hexanoate	40	40											
D-Limonene	40	40	40		40	40	40		40		40		40
β-Phellandrene	40	40											
Hexanol	4	4	4	4	4	4	4	4		4			4
γ-Terpinene	40												
cis-3-Hexen-1-ol	4	4	4	4	4	4	4	4	4	4	4	4	
1,8-Cineole													
Ethyl hexanoate	4	4	4	4	4	4	4	4	4	4		4	4
trans-2-Hexen-1-ol	4	4											
p-Cymene													

ciates) with a Resolve silica Radial-PAK HPLC cartridge (5-μm particle size) was used. Mobile phase was hexane at 2 ml/min. Purity of the HPLC-purified α-terpinene was determined by GC. Three mixtures were prepared. Mixes 3G and 2C were repeated. Mix 3G contained commercial α-terpinene (88% pure). Mix 3H was prepared like mix 3G except purified α-terpinene (99.7%) was used.

Mixture experiment 7 was conducted after results of mixture experiment 6 dictated that contaminants in commercial-grade α-terpinene be identified. GC-MS of commercial α-terpinene identified 1,8-cineole and *p*-cymene, among others. 1,8-Cineole was obtained (Table 1). Mix 3I contained *p*-cymene at 2 ng/μl to reflect its concentration in commercial α-terpinene. Mix 3K contained 1,8-cineole at 4 ng/μl to reflect total cineole (1,4-cineole and 1/8-cineole) found in commercial α-terpinene. The purposes of mixes 3I and 3K were to evaluate *p*-cymene and 1,8-cineole, respectively, as the active chemicals in commercial

TABLE 2. Continued

Mix														
4E	3A	3B	3C	3D	3E	3F	3G	3H	3I	3J	3K	2A	2B	2C
			40							40				
	40	40	40	40		40	80	80				40	40	
				40	40	40							40	
4	4				4	4	4	4	4	4	4			4
		4												
4											4			
4	4	4	4	4	4		4	4	4	4	4	4		4
2									2					

α-terpinene. Mix 3J was tested to evaluate (+)-sabinene in combination with hexanol and ethyl hexanoate since this had not been done earlier.

Mixture experiment 8 was conducted to evaluate the ability of *p*-cymene to augment the attractiveness of mix 3K. Mix 4E equaled mix 3K plus *p*-cymene at 2 ng/μl. In addition, 1,8-cineole was tested individually at 4 ng/μl, during this experiment.

Ratio Experiment. This experiment was to determine the most attractive ratios of 1,8-cineole, ethyl hexanoate, and hexanol. Eight mixtures were prepared, each containing all three chemicals. Ratios were 1:1:1, 10:1:1, 1:10:1, 1:1:10, 10:10:1, 10:1:10, 1:10:10, and 10:10:10, of 1,8-cineole, ethyl hexanoate, and hexanol, respectively. The 1:1:1 mixture equaled mix 3K. In all mixtures, "1" indicated 4 ng/μl and "10" indicated 40 ng/μl. The ratio mixtures were tested as in the other mixture experiments.

Dose–Response Experiment. Eight concentrations of the 10:1:1 mixture

TABLE 3. ATTRACTIVENESS TO *A. ludens* OF VARIOUS MIXTURES OF CHEMICALS SUMMED OVER TWO TEST QUANTITIES IN EIGHT EXPERIMENTS[a]

Mixture	Mean RI	Mixture	Mean RI
CVSM 10 μl	4.7 ab	Exp. 4	
CVSM 100 μl	6.4 b	4D	5.5 d
CV 10 μl	3.5 a	3A	4.6 cd
Exp. 1		3E	1.6 a
15	5.0 a	3F	4.1 c
12	5.0 a	2B	3.9 bc
8	6.4 a	2C	2.8 b
7A	5.4 a	Exp. 5	
7B	5.4 a	4D	5.8 a
7C	5.7 a	3G	6.4 a
Exp. 2		Exp. 6	
6	7.7 c	3G	4.7 b
5A	5.9 bc	3H	3.6 a
5B	5.6 b	2C	4.1 ab
4A	5.8 b	Exp. 7	
4B	3.4 a	3I	4.5 a
4C	6.3 bc	3J	4.4 a
Exp. 3		3K	5.9 b
4D	6.0 b	2C	4.1 a
3A	6.2 b	Exp. 8	
3B	6.0 b	4E	6.0 a
3C	3.6 a	3K	5.9 a
3D	3.8 a		
2A	3.2 a		

[a] Mean RIs within the same experiment followed by the same letter are not significantly different from each other at the 5% level by LSD.
[b] Responses are expressed relative to attractiveness of 100 μl of CVSM.

of 1,8-cineole, ethyl hexanoate, and hexanol were prepared. The lowest concentration contained 1,8-cineole at 0.04 ng/μl and ethyl hexanoate and hexanol each at 0.004 ng/μl. Each concentration increased by a factor of 10 to the highest concentration that contained 1,8-cineole at 400 μg/μl and ethyl hexanoate and hexanol each at 40 μg/μl. The mixtures were tested only at the 10-μl quantity using the cage-top bioassay.

Emissions Tests. The quantities were determined of chemicals emitted during a 5-min period from filter papers loaded with 40 μg of 1,8-cineole, and 4 μg each of ethyl hexanoate and hexanol in 10μl of hexane. Each filter paper (3 × 3 cm) was placed into a 500-ml round-bottom flask with a ground-glass stopper immediately after loading with the chemicals. After 5 min, the filter paper was discarded and the flask rinsed with 10 ml of 10% acetone in pentane. The emissions were analyzed by GC. Five replications were conducted.

As a check for sample handling losses, a test similar to the one described above was conducted in which the 10 μl of test mixture was injected directly into a 500-ml round-bottom flask without filter paper. Five replications were conducted.

For another emissions test, a 10 : 1 : 1 (w/w/w) mixture of 1,8-cineole, ethyl hexanoate, and hexanol (CEH) was prepared neat. Either 25 or 100 μl of the CEH mixture was put into a 400-μl polyethylene microcentrifuge tube (Centaur West, Inc., Sparks, Nevada, Catalog #219-OMT1-006) and closed with the attached cap. The tube was put into a 500-ml round-bottom flask with a ground-glass stopper for 0.5 hr, then transferred to a second flask for 0.5 hr. Each flask was extracted immediately after removal of the tube. Five replications were conducted for both 25- and 100-μl quantities.

YB/CEH Comparison. The purpose of this experiment was to compare the attractiveness of CEH to an aqueous solution of *Torula* dried yeast and borax (YB), a common bait used for detection of *A. ludens* and other *Anastrepha*. YB traps were prepared using six *Torula* yeast–borax "bait pellets" (Sit-Khem Corp., Michigan City, Indiana) in 300 ml of water in a McPhail trap (Baker et al., 1944). The YB was allowed to ferment for four to five days before tests. CEH was prepared neat and either 25 or 100 μl were put into a polyethylene microcentrifuge tube as described above (Emissions Tests). The tubes were suspended in the entrance holes on the undersides of McPhail traps containing 300 ml of amber-colored water to mimic the color of YB. Amber-colored water was prepared from red, yellow, and green McCormick food colors (McCormick & Co., Inc., Baltimore, Maryland). The amber-colored water also contained 0.01% Triton X-100 (Rohm and Haas Co., Philadelphia, Pennsylvania) as a wetting agent.

The traps were tested in an aluminum-screened wind tunnel (2.0 m long × 0.7 m wide × 1.3 m high) with an airflow of 1–2 m/sec in a greenhouse. Approximately 800–1000 2-day-old flies were released into the downwind end of the cage at the beginning of each replication. The traps were removed from the cage after 1–2 hr. Four replications using 25 μl of CEH and five using 100 μl were conducted.

Statistical Analyses. Tandem GC–behavior bioassays of CV and CVSM were analyzed by paired *t* tests comparing tops of behavior peaks to baseline. All data were smoothed by averaging with the points from the preceding and following 15-sec periods. Thus, peak tops and baselines were lower and higher, respectively, than the actual data. Variances were calculated from unsmoothed data.

Individual chemical tests were analyzed by paired *t* tests to compare total counts beneath the solvent-control papers to counts beneath chemical-loaded papers. Mixture experiments 1–8 and the dose–response experiment were randomized complete block designs and were analyzed by separate analyses of variance (ANOVA). Means were compared by the least significant difference

method. The ratio experiment was a randomized complete block design and was analyzed by a factorial ANOVA with three factors (chemicals). Captures in McPhail traps containing CEH and YB were compared by paired *t* tests.

RESULTS AND DISCUSSION

Identification of Chemicals in CV. Over 70 chemicals were identified by GC-MS. The 10 most abundant chemicals were: germacrene-D, 52% of the total chemicals in CV; caryophyllene, 9.2%, α-pinene, 6.6%; unknown, 4.4%; β-bourbonene, 3.2%; humulene, 1.8%; alloaromadendrene, 1.7%; γ-cadinene, 1.5%; limonene, 1.5%; and unknown, 1.5%.

Tandem GC–Behavior Bioassays of CV and CVSM. The 5 to 15-min retention time region was the most attractive region of the CV chromatogram, containing three distinct attraction peaks (each $P < 0.001$). The 28 chemicals in Table 1 were identified from this region by GC-MS (except that optical stereoisomerism was not determined). In addition to α-pinene and limonene discussed above, major components in the 5 to 15-min region were: γ-terpinene, 1.4% of the total; *p*-cymene, 1.2%; and β-myrcene, 1.1%. Five smaller attraction peaks in the 20 to 60-min retention time region were also significantly greater than baseline ($P < 0.05$). This region was not investigated. Only one of the three attraction peaks elicited by CV in the 5 to 15-min region was reproduced by CVSM. Apparently, some unknown chemicals in CV but absent from CVSM were responsible for the other two attraction peaks.

Tests of Individual Chemicals. Figure 1 shows that 11 chemicals were significantly attractive in at least one of the two test quantities and five others were significantly attractive summed over both test quantities. In general, short-chain, oxygenated molecules were more attractive at the lower odor concentration. These included pentanol, hexanol, ethyl hexanoate, and *trans*-2-hexen-1-ol. Exceptions were methyl hexanoate and *cis*-3-hexen-1-ol, which elicited nearly equal responses at the two concentrations. Terpenoid molecules were more attractive at the higher concentration. These included (+)-sabinene, α-terpinene, D-limonene, and γ-terpinene. 1,8-Cineole could not be compared since it was not tested at the same two concentrations as the other chemicals. Also, note that because α-terpinene contained 3% 1,8-cineole (see Mixture Experiments, below), the apparently greater response to the higher concentration of α-terpinene may have been response to 1,8-cineole (120 ng in the 100-μl sample of α-terpinene).

Mixture Experiments. The results of the mixture experiments, summed over low and high test quantities, are shown in Table 3. Summing over low and high quantities was done because they were equally attractive in all but two cases. The exceptions were: mix 4C ($P < 0.05$, $t = 2.3$, $df = 18$) and mix 3B ($P <$

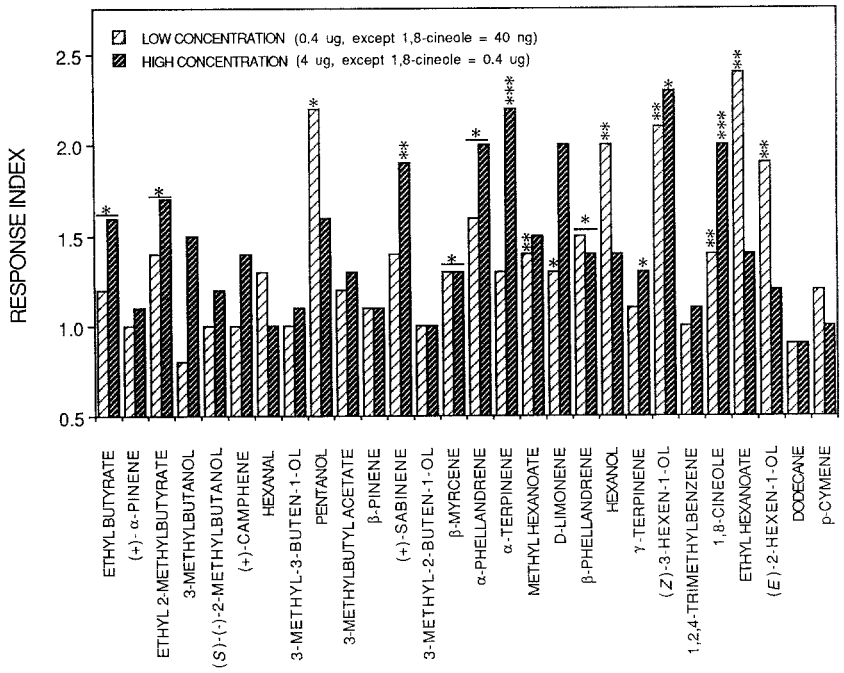


FIG. 1. Attractiveness to *A. ludens* of individual chemicals identified from chapote volatiles extract, at two odor concentrations. Attractiveness significantly higher than blanks by paired t-tests is indicated as: $*P < 0.05$; $**P < 0.01$; and $***P < 0.001$. * placed above a bar refers to that bar while * placed above a line refers to the average of the high and low test quantities.

0.05, $t = 2.7$, $df = 18$). In each case, the higher quantity was more attractive than the lower. Because these were isolated cases, the results were regarded as random variation and were not investigated further.

The mean RI for 100 μl of CVSM was 6.4. This was not significantly higher than the response to 10 μl of CVSM but was higher than the response to 10 μl of CV. This indicates that the synthetic mixture CVSM at least accounts for the activity of CV.

All mixtures in experiments 1 and 2 were nearly equivalent. One exception was the low attractiveness of mix 4B, the first mixture tested that did not contain ethyl hexanoate. In experiment 3, mixes 3C, 3D, and 2A, all not containing hexanol or another short straight-chain alcohol, were less attractive than the other mixtures containing either hexanol or *cis*-3-hexenol. In experiment 4, mix 3E without α-terpinene (88%), 3F without ethyl hexanoate, 2B without a short

straight-chain alcohol, and 2C without a terpenoid component, were less attractive than 4D and 3A, both of which contained ethyl hexanoate, hexanol, and α-terpinene (88%). Results to this point indicated that an alcohol such as hexanol, an ester such as ethyl hexanoate, and a terpene such as α-terpinene were necessary and sufficient components of the minimum-component mixture that retained the attractiveness of the original 15-component mixture (mix 15).

Several questions remained. Did D-limonene contribute to the attractiveness of mix 4D since it was slightly more attractive (not significant) than mix 3A? In experiment 5, mix 3G containing extra α-terpinene (88%) to replace D-limonene was at least as attractive as mix 4D containing both α-terpinene (88%) and D-limonene. Thus D-limonene was eliminated from further consideration.

Mix 3G, the minimum-chemical mixture with attractiveness equal to CVSM, contained commercial grades of ethyl hexanoate, hexanol, and α-terpinene (Table 1). Investigation by GC-MS showed that ethyl hexanoate and hexanol each contained no contaminants greater than 0.5%. Commercial-grade α-terpinene contained: α-terpinene, 88.3%; β-myrcene, 0.4%; α-phellandrene, 1.4%; D-limonene, 1.7%; *p*-cymene, 2.9%; 1,4-cineole, 1.6%; 1,8-cineole, 3.0%; and unidentified components, 0.7%. α-Terpinene was purified by HPLC to 99.7%.

In mixture experiment 6, mix 3H, containing purified α-terpinene, was less attractive than mix 3g, containing commercial grade α-terpinene. We concluded that α-terpinene was not responsible for the attractiveness of commercial α-terpinene in mix 3G.

In mixture experiment 7, mix 3K, containing 1,8-cineole, ethyl hexanoate, and hexanol, was ca. 1.4 times more attractive than mix 2C, containing only ethyl hexanoate and hexanol. Mixes 3I and 3J containing *p*-cymene and (+)-sabinene, respectively, in addition to ethyl hexanoate and hexanol, were not more attractive than mix 2C. We concluded that 1,8-cineole was the contaminant of commercial grade α-terpinene responsible for part of the attractiveness of mix 3G and that neither *p*-cymene nor (+)-sabinene contributed to the attractiveness of mix 3G.

In mixture experiment 8, mixes 4E and 3K were equivalent, indicating the *p*-cymene did not increase the attractiveness of a mixture containing 1,8-cineole, ethyl hexanoate, and hexanol. *p*-Cymene was eliminated from further consideration.

Reexamination of CV by GC-MS. Careful reexamination of CV found 1,8-cineole present as a trace component. The concentration was ca. 0.5 times that of α-terpinene. 1,4-Cineole was not found.

Ratio Experiment. Only small differences in attractiveness of the eight mixtures occurred despite a wide range of chemical ratios (Figure 2). However, the 10:1:1 ratio was chosen for further testing for several reasons. First, the four

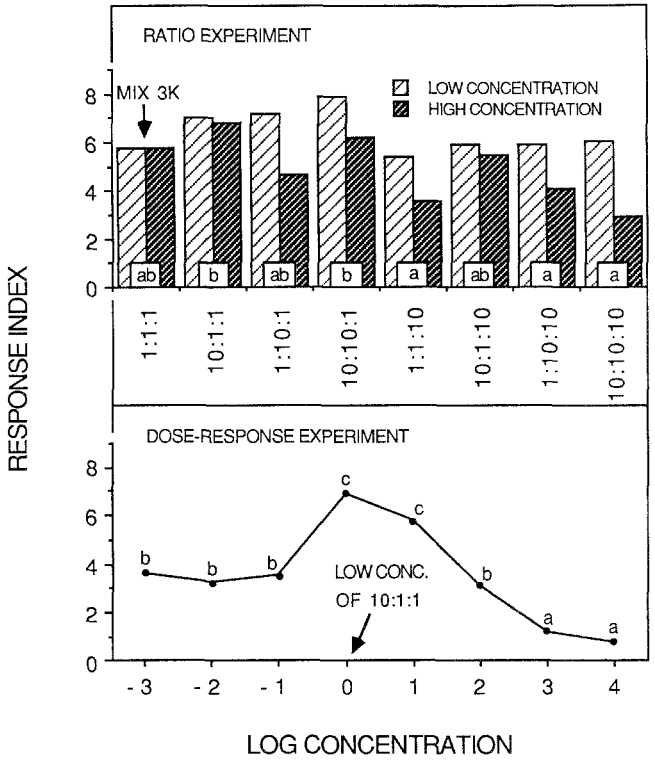


FIG. 2. Ratio experiment: Attractiveness to *A. ludens* of mixtures containing various ratios of 1,8-cineole, ethyl hexanoate, hexanol, at two concentrations. Dose–response experiment: Mixtures within the same experiment with the same letter are not significantly different from each other at the 5% level by LSD.

mixtures containing hexanol at the relative concentration of "10" were eliminated because of generally lower attractiveness than the four mixtures with hexanol at "1." Of the remaining four mixtures, the two containing ethyl hexanoate at "10" were eliminated because the 100-μl quantities were less attractive than the 10-μl quantities (each $P < 0.05$). Finally, the 1:1:1 mixture was eliminated based on consistently greater attractiveness of the 10:1:1 mixture at both test quantities (not significant).

Dose–Response Experiment. The most attractive mixtures were the two designated as log concentrations 0 and 1 (Figure 2). These concentrations represented 400 ng and 4 μg of 1,8-cineole, and 40 and 400 ng each of ethyl hexanoate and hexanol, respectively, per filter paper. Log concentrations 0 and 1 equaled the low and high concentrations, respectively, of the 10:1:1 ratio

tested in the ratio experiment (Figure 2). Attractiveness decreased rapidly as log concentration increased to 2, 3, and 4. Attractiveness of log concentration −1 was about half that of log concentration 0 ($P < 0.05$), but no further decrease in attractiveness occurred as log concentration decreased to −2 and −3. These results demonstrate that too much of this mixture is not attractive to the flies, while amounts less than optimum retain moderate attractiveness. We concluded that the 10 : 1 : 1 mixture of 1,8-cineole, ethyl hexanoate, and hexanol at 400 ng–4 μg, 40–400 ng and 40–400 ng, respectively, applied to filter papers, is the optimal mixture of these chemicals, at least under our conditions.

The log concentration 0 mixture (Figure 2) was 1.1 and 2.0 times more attractive than 100 μl of CVSM and 10 μl of CV, respectively (Table 3). Note, however, that greater quantities of CVSM and CV that may have been more attractive were not tested. It also is possible that other potentially attractive chemicals are present in CV but in concentrations too low to elicit responses.

Receptor Site Physiology. The attractivenesses of 1,8-cineole, ethyl hexanoate, and hexanol were additive. The high concentration of mix 3K, consisting of 400 ng each of the three chemicals applied to filter paper, elicited RIs of 5.8 in the ratio experiment, 5.9 in mixture experiment 7, and 6.5 in mixture experiment 8 for a mean RI of 6.1. In separate tests, 400 ng of each of the three individual chemicals elicited RIs of 2.0, 2.4, and 2.0 for 1,8-cineole, ethyl hexanoate, and hexanol, respectively (Figure 1). The sum of these RIs, 6.4, is nearly equal to the RI elicited by the three-chemical mixture (6.1). Further, the high concentration of mix 2C, consisting of 400 ng each of ethyl hexanoate and hexanol, elicited RIs of 3.6 in mixture experiment 4, 3.5 in mixture experiment 6, and 4.0 in mixture experiment 7 for a mean RI of 3.7. This RI is nearly equal to the sum of the RI's of its components, 2.4 + 2.0 = 4.4. Note, however, that these additivity comparisons are only approximations, since RIs are not defined in a way that allows them to be summed.

These results cannot be explained simply as addition of more molecules stimulating the same receptor site. If that were true, then greater quantities of the chemicals would also elicit higher RIs. In fact, greater quantities of two of the chemicals, ethyl hexanoate and hexanol, elicited lower RIs than the smaller quantities (Figure 2). Instead, the results indicate the presence of three receptor types, each maximally sensitive to different molecule classes represented in this work by 1,8-cineole, ethyl hexanoate, and hexanol. Apparently, stimulation of each receptor type independently increases attraction behavior. Quite possibly, more receptor types sensitive to other chemical classes remain to be discovered in *A. ludens*.

Emissions Tests. The amounts emitted from filter papers during 5 min were 78, 75, and 40% of the 1,8-cineole, ethyl hexanoate, and hexanol, respectively, loaded onto the papers. We predicted that at least these percentages, possibly up to 100%, would emit from filter papers loaded with 1 or 10% as much of

the chemicals, the amounts that were most attractive to flies in the dose–response experiment (log concentrations 0 and 1). The predicted emissions for 5-min periods were converted to a 0.5-hr basis by multiplying by six. Thus, the predicted emission ranges covering the two most attractive concentrations were 1.9–24, 0.18–2.4 and 0.10–2.4 μg/0.5 hr for 1,8-cineole, ethyl hexanoate, and hexanol, respectively. These amounts were predicted rather than measured because of detection problems at the low emission rates.

Quantities emitted from polyethylene tubes during both 0.5-hr periods generally were in the attractive ranges predicted above. One exception was the first 0.5 hr of emission from the polyethylene tubes containing 25 μl of CEH. Even in this case, however, three of the five tubes emitted within the predicted attractive ranges of the three chemicals. There were no significant differences between the 25- and 100-μl tubes. The mean emissions from the tubes during the first 0.5 hr (summed over 25- and 100-μl amounts) were: 1,8-cineole, 10.1 $\pm$ 4.2 μg (mean $\pm$ SE); ethyl hexanoate, 3.3 $\pm$ 1.7 μg; and hexanol, 5.8 $\pm$ 4.0 μg.

An important point is that filter papers used in cage-top bioassays emitted the chemicals in relatively still air while polyethylene tubes used in the greenhouse wind tunnel emitted chemicals in moving air. Thus, our predictions of attractive emission ranges of the chemicals, based on measurements in still air, should not be regarded as absolute.

High variability of emission was characteristic of the polyethylene tubes where standard errors averaged 64% of the means. The variability was attributed to the tubes instead of the extraction procedure since standard errors of the checks and filter paper emissions averaged only 10 and 16% of their respective means.

Emissions from polyethylene tubes during the second 0.5 hr averaged 26% of the first 0.5-hr emission. This result, coupled with the great variability of emission among tubes discussed above, demonstrates that research is needed to develop a dispenser that emits a reliable quantity of the chemicals for a long period of time.

YB/CEH Comparison. Table 4 shows that CEH attracted ca. 1.8 times as many starved, 2-day-old, male and female flies as YB, when both baits were tested in McPhail traps in a cage in a greenhouse ($P < 0.001$). The 25- and 100-μl quantities of CEH performed equally. Robacker et al. (1990) showed that fermented chapote fruit was 3.6 times more attractive than YB in greenhouse experiments. This suggests that fermented chapote fruit contains additional chemicals that could enhance the attractiveness of CEH.

The results of this work suggest that CEH may be a more potent field attractant than YB for *A. ludens*. However, CEH was developed using starved, 2-day-old, laboratory-culture flies as test animals. Responses of older flies, wild flies, and unstarved flies have not been evaluated. Robacker et al. (1990) showed that laboratory flies of various ages, starvation levels, and mating status were

TABLE 4. CAPTURES OF 2-DAY-OLD, NEVER-FED ADULTS OF *A. ludens* BY COMPETING PAIRS OF MCPHAIL TRAPS BAITED WITH NEAT MIXTURE OF 1,8-CINEOLE, ETHYL HEXANOATE, AND HEXANOL (CEH) (10:1:1) VS. AQUEOUS SOLUTION OF *Torula* DRIED YEAST AND BORAX (YB) IN WIND TUNNEL IN GREENHOUSE[a]

Bait	Number of replications	Males[b]	Females[b]	Males + females[b]	CEH/YB (M + F)
25 μl CEH	4	44	40	42	1.8
YB		25	22	23	
100 μl CEH	5	45	40	43	1.8
YB		25	23	24	
Overall CEH	9	45b	40b	43b	1.8
YB		25a	23a	24a	

[a] CEH traps contained 300 ml of amber-colored water to mimic YB color; YB traps contained 300 ml of water + 6 *Torula* yeast bait pellets.
[b] Data are mean percentages of the total flies in the chamber captured by each trap. Means in the same vertical column followed by different letters are significantly different at the 0.1% level by paired *t* tests.

attracted to chapote odor, but responses were not compared with responses to YB.

Another important point is that the comparison of CEH with YB was conducted with gamma-irradiated flies. Galun et al. (1985) showed that attractiveness of yeast hydrolysate to two tephritid species irradiated with gamma rays was 50% less than to unirradiated flies. Similarly, Robacker et al. (1990) found that gamma irradiation adversely affected responses of *A. ludens* females to chapote odor. Thus, irradiation of flies probably lowered responses to both YB and CEH, but perhaps more so to YB because of a probable link between sterility, need for protein, and attractiveness of proteinaceous baits such as YB (Galun et al., 1985).

Finally, CEH-baited McPhail traps used in the present work also contained amber-colored water. The purpose was to equalize factors other than odor differences. Under dry climatic conditions, water may be an important component of the attractiveness of YB. In one study, traps containing proteinaceous baits in water were 20 times more attractive to *Dacus dorsalis* in dry than in wet climates (Cunningham et al., 1978). Water also may be necessary in CEH-baited traps to make them competitive with YB. In our tests, attractiveness of water was minimized by giving flies access to water before tests. Nevertheless, it has not been shown that CEH will attract flies in traps without water.

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SALIVARY GLANDS AND PREPUTIAL GLANDS OF MALES AS SOURCE OF ESTRUS-STIMULATING PHEROMONE IN FEMALE MICE

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Abstract—Male mice produce pheromones that induce estrus in adult female mice. The synthesis of these chemosignals is controlled by testosterone. Male tissues abundant in testosterone-binding receptors were tested for their ability to stimulate estrus in grouped females. The percentage of females that showed estrus during two days following exposure to male tissues was used as the indicator of biological activity of tested samples. Homogenates of salivary glands and preputial glands induced estrus; other tested organs (liver, testes, coagulation and vesicular glands or kidneys) did not stimulate estrus in female mice. These results indicate that estrus-stimulating pheromone has multiple sources.

Key Words—Chemosignal, pheromone, estrus cycle, preputial glands, salivary glands, laboratory mice, *Mus musculus domesticus*.

INTRODUCTION

The stimulation of ovulation in adult female mice by the presence of male mice was the first described pheromonal effect in mammals (Whitten, 1956). Male chemosignals also accelerate puberty of female mice (Vandenbergh, 1967) and block pregnancy (Bruce, 1959). These chemosignals are present in male urine and their production is under androgen control (Dominic, 1965; Bronson and Whitten, 1968; Vandenbergh, 1969). Very little is known about the chemical structure and the site of synthesis of male pheromones.

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Since the synthesis of male pheromones is controlled by testosterone, male tissues abundant in testosterone-binding receptors were tested for their ability to stimulate the estrus cycle in female mice. The percentage of females showing estrus during the two days following exposure to male pheromone was used as the indicator of biological activity of tested samples. Bronson (1971), using a similar bioassay, showed that the percentage of wild and SJL/J female mice that exhibited estrus depends on the concentration of applied male urine.

METHODS AND MATERIALS

Adult virgin female and adult male mice (*Mus musculus domesticus*) from outbred stock bred in our laboratory (maintained as a closed population with maximum avoidance of inbreeding) were used for the experiments. They were maintained in polyethylene cages (18 × 15 × 10 cm), with a lighting schedule of 14:10 (lights on 7:00 AM to 9:00 PM) and fed a standard pelleted diet of Murigran (Bacutil, Motycz, Poland) and water. The ground wood shavings used for bedding was changed weekly.

Experiment 1. This experiment was designed to assess the sensitivity of our outbred females to male chemosignals. Urine of outbred males was collected by holding the animals over a test tube and gently massaging the abdominal wall. Urine was kept frozen at −15°C until used. Just before testing, the urine was thawed and various concentrations were prepared: 0.125 ml urine + 0.375 ml physiological saline; 0.250 ml urine + 0.250 ml physiological saline; 0.5 ml or 1.0 ml urine.

Experiment 2. To investigate the presence of estrus-stimulating chemosignals in male tissues, 237 males (2–5s month old, body weight 31.8 + 0.3 g) were killed and the following organs were dissected and weighed: sublingual salivary glands, submandibular salivary glands, and parotid glands were removed together (288.1 ± 5.0 mg); preputial glands (130.9 ± 2.8 mg); coagulation glands and vesicular glands dissected together (252.9 ± 6.7 mg); the liver (1790.0 ± 132.1 mg); the brain (376.5 ± 6.7 mg); and pairs of testes (233.0 ± 4.0 mg); and kidneys (563.6 ± 11.8 mg). These organs were immediately homogenized with physiological saline (200 mg tissue/0.5 ml) centrifuged for 5 min at 3000 g and the supernatant stored at −15°C until used.

Bioassay. For the bioassay, 470 outbred females were isolated from males starting from the 24th day after birth. Virgin 8- to 10-week-old females were grouped (10 females per cage) and transferred to a male-free room with changed photoperiod (lights on noon to 2:00 PM).

The light schedule was changed because female mice are more sensitive to male chemosignals during the dark phase (Marchlewska-Koj and Kruczek, 1986). The mice were allowed two weeks to adapt to the new light schedule.

Afterwards each female was transferred to a separate plastic box (9 × 9 × 7 cm) at 10:00 AM and exposed to tested samples for 2 hr. This part of the experiment was performed under red lights. The mouse was removed and returned to its own cage at noon. The females were exposed to test samples for four consecutive mornings. Test samples were delivered to the plastic cages just before the females were placed in the cage.

The estrous state of the female was estimated during two consecutive days between noon and 1:00 PM: the last day of the exposure and the first day following exposure to tested samples. Vaginal smears were taken and immediately examined under the microscope. Females that showed a swollen vagina and only numerous cornified epithelial cells in the smear were regarded as being in estrus. The percentage of females showing estrus during these two days was taken as the indicator of biological activity of the tested samples.

RESULTS

As shown by the results summarized in Table 1, the exposure of grouped outbred female mice to male urine stimulated estrus in the tested females. The reaction of females was dependent on the dose of tested male urine. Only the presence of male-soiled bedding or 0.5 ml of male urine evoked a statistically significant increase of the number of females in estrus. Higher or lower doses of urine were less effective. The results indicate that a proper concentration of olfactory stimulants is required to evoke estrus in outbred female mice. Bronson (1971) reported that an excessive dose of male urine reduced the maximum

TABLE 1. NUMBER OF FEMALE MICE IN ESTRUS AFTER EXPOSURE TO MALE-SOILED BEDDING OR VARIOUS DOSES OF MALE URINE

Treatment (female/day)	Females (*N*)		Females in estrus (%)
	Tested	In estrus	
Male bedding 10 ml	40	27	67.0a
Physiological saline 0.5 ml	40	12	30.0a,b
Male urine (ml):[a]			
0.125	40	10	25.0
0.250	39	13	33.3
0.500	40	29	72.5b
1.000	20	7	35.0

[a] For further details see Methods and Materials. Data were analyzed by χ^2 test: a, physiological saline vs. male bedding, $P < 0.05$; b, physiological saline vs. 0.5 ml of male urine, $P < 0.05$.

TABLE 2. NUMBER OF FEMALE MICE IN ESTRUS AFTER EXPOSURE TO 0.5 ml EXTRACT OF HOMOGENIZED SALIVARY GLANDS, PREPUTIAL GLANDS, COAGULATION AND VESICULE GLANDS, LIVER, TESTES, KIDNEYS, OR BRAIN[a]

Treatment (0.5 ml/ female/day)	Females (N)		Females in estrus (%)
	Tested	In estrus	
Physiological saline	30	7	23.3a,b
Salivary glands	30	17	56.7a
Preputial glands	40	22	55.0b
Coagulation and vesicule glands	30	10	33.3
Liver	30	8	26.7
Testes	30	11	36.7
Kidneys	30	9	30.0
Brain	30	8	26.7

[a] Data were analyzed by χ^2 test: a, physiological saline vs salivary glands, $P < 0.05$; b, physiological saline vs. preputial glands, $P < 0.05$.

response in females from different strains of mice and in deer mouse females. Olfactory pregnancy termination (Bruce effect) in female mice exposed to a double concentration of urinary protein fraction salted out from male urine also was less effective than in females exposed to a physiological level of these proteins (A. Marchlewska-Koj, unpublished observations).

On the basis of these observations, 0.5 ml of homogenized tissues were tested for the ability to stimulate estrus in female mice. The results summarized in Table 2 show that the presence of extracts of salivary glands or preputial glands increased the percentage of females exhibiting estrus. In contrast, other tested organs (liver, testes, coagulation and vesicular glands, or kidneys) did not affect the number of females in estrus.

DISCUSSION

The protein fraction salted out from male urine synchronized the estrus cycle in mice, as did the presence of a male behind a screen (Marchlewska-Koj and Biały, 1978). A urinary protein fraction accelerated puberty (Vandenbergh et al., 1975) and evoked the pregnancy block effect (Marchlewska-Koj, 1977). The major urinary proteins (MUPs) are the main protein fraction of mouse male urine (Finlayson et al., 1965). The MUPs are synthesized in the liver and

secreted into the circulation and then excreted with the urine (Szoka et al., 1980). Significant levels of MUP mRNA were found not only in the liver but also in the salivary glands of male mice (Shaw et al., 1983). However, neither MUP nor its mRNA were detected in the preputial glands of male mice (Held and Gallagher, 1985).

The present results indicate that an extract of the liver failed to stimulate females, whereas salivary gland and preputial gland extracts stimulated estrus in females. The lack of positive reaction of tested females to the homogenates of liver, kidneys, or testes does not exclude these organs as the source of male pheromone. The chemosignals could be synthesized and not retained in tissues but secreted into the blood and excreted into male urine.

Acceleration of puberty, synchronization of the estrus cycle, and pregnancy block effect can be evoked by urine collected directly from the bladder and thus free of any accessory gland secretion (Bronson and Whitten, 1968; Marchlewska-Koj, 1977; Drickamer and Murphy, 1978). Furthermore, the hemizygous Tabby males with an inherited lack of preputial glands evoked the pregnancy block effect (Hoppe, 1975), and preputialectomized as well as intact males accelerated the first estrus in juvenile females (Colby and Vandenbergh, 1974).

In mice, the preputial glands are known as the source of androgen-dependent olfactory stimulants such as an aggression-promoting pheromone (Mugford and Nowell, 1971) or a sex attractant (Bronson and Caroom, 1971). These glands also contain non-androgen-dependent chemosignals that attract female mice (Ninomiya and Kimura, 1988). The present results confirm the previous finding that biologically active substances that accelerate estrus (Gaunt, 1968; Chipman and Albrecht, 1974) are present in the preputial glands. They also provide additional evidence that the estrus-stimulating pheromone has multiple sources.

The presence of the male is more effective in acceleration of puberty of juvenile females (Bronson and Maruniak, 1975) or stimulation of the estrus cycle in adult females (Marchlewska-Koj and Biały, 1978) than exposure to male urine. Milligan (1974, 1975) also found that in voles (Microtus agrestis) ovulation may be induced by close proximity of a male. The effectiveness of the male was reduced by increasing the degree of separation from the female. However, ovulation may still occur after exposure of the female to just male-soiled bedding. The finding that salivary glands are an additional source of male olfactory signals may help to explain why direct contact with the male evokes a more pronounced effect than exposure of a female to male urine.

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SQUIRTING AND REFILLING: DYNAMICS OF *p*-BENZOQUINONE PRODUCTION IN DEFENSIVE GLANDS OF *Diploptera punctata*[1]

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Abstract—*Diploptera punctata*, a Pacific islands cockroach, discharges a fine aerosol of *p*-benzoquinones from a pair of tracheal glands in response to disturbance or CO_2 anesthesia. In addition, the glands and their contents are shed at each molt. We measured the amount of *p*-benzoquinones discharged in response to pinching and anesthesia and the filling of glands after discharge and molting. Roaches discharge highly variable amounts of quinones but appear to retain approximately 11% of their lifetime total benzoquinone content after repeated discharges. Roaches rapidly fill their glands after molting (18 μg quinone/day) but after discharging appear to refill at a much slower rate or not at all. This lack of refilling could result from an inability to produce quinones after a finite time period or in excess of a fixed quantity. The delayed accumulation of *p*-benzoquinone with respect to the alkylated derivatives in newly molted adults suggests competition for aromatic amino acids between cuticular and defensive quinone synthesis.

Key Words—*Diploptera punctata*, Orthoptera, Blaberidae, cockroach, benzoquinone, quinone, chemical defense.

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INTRODUCTION

Many insects discharge defensive secretions in response to attack or disturbance (Eisner, 1970; Bettini, 1978; Blum, 1981). Little is known about the operational dynamics of such glandular systems—parameters such as the synthesis, storage, and release of defensive secretions. These parameters are critical to an understanding of the phenotypic costs of secretory production and the benefits of defensive use. Secretory production is known to vary seasonally and with diet in some species (Wheeler et al., 1970; Fescemyer and Mumma, 1983; Jones et al., 1988), but important determinants of the defensive potential of discharged chemicals—for example, the amount sprayed, the proportion of the reservoir that is sprayable, and refilling rates of the reservoir—remain unexplored in all but a few cases (Fescemyer and Mumma, 1983).

Diploptera punctata (Eschscholtz), a viviparous roach from the Pacific islands, has a pair of abdominal tracheal glands that produce a mixture of *p*-benzoquinones (Roth and Stay, 1958; Brossut, 1983). The glands are associated with the second pair of abdominal spiracles, and the secretion is forcibly discharged through these spiracles when the animal is attacked; the resulting spray is a proven defense against ants, carabid beetles, and frogs (Eisner, 1958; Eisner et al., 1959). As a rule, the roaches discharge only from the gland of the side attacked, but they are capable of discharging from both glands simultaneously and can do so a number of times in succession (Eisner, 1958). The glands contain a mixture of three *p*-benzoquinones (*p*-benzoquinone, methyl-*p*-benzoquinone and ethyl-*p*-benzoquinone) as the major components (Roth and Stay, 1958), with tiglic and methylacrylic acids as minor components (Brossut, 1983). The gland contents are discharged as a fine aerosol spray when air is forced from the tracheae into the quinone-containing glandular reservoirs. Hence, once discharged, the chemical defense is lost to the roach and must be resynthesized. Moreover, the cuticular gland and its contents must be replaced after each molt. Freshly molted adults are without chemical defense but have been observed to spray 24 hr after molting (Eisner, 1958), suggesting that the chemical stockpile can be replenished quickly.

Here we quantify the amounts of quinone sprayed and the rates of refilling of glands after both molting and discharge in individual roaches. Clearly, the defensive properties of the chemical defense depend not only on the amount sprayed, the sensitivity of the attacker to the spray, and the speed with which the roach can refill its quinone supplies, but also on the chemical composition of the discharge (Jones et al., 1988). The three benzoquinones present in the gland differ in their repellency and irritancy to insects in standardized assays (Peschke and Eisner, 1987), indicating the importance of the composition of the spray to its defensive potential.

METHODS AND MATERIALS

Source and Maintenance of Roaches. Diploptera punctata originated from a laboratory colony established in 1957 and maintained mostly on dry dog food and water.

Quantification of Quinones. Extracts of roach quinones were analyzed on a Hewlett Packard 5890 gas chromatograph equipped with flame ionization detection, and quantified with a Hewlett Packard 3390 integrator. Quinones were separated on a 13.2-m (0.53 mm ID) bonded-phase methyl silicon column with H_2 carrier gas at a flow of 110 cm/sec and had the following retention times: *p*-benzoquinone 0.9 min; methyl-*p*-benzoquinone 1.6 min; ethyl-*p*-benzoquinone 3.0 min; 2,6 dimethyl-*p*-benzoquinone 3.2 min. Analysis was performed isothermally at 65°C with detector and injector at 250°C and 200°C, respectively. Relative response factors between the internal standard (2,6 dimethyl-*p*-benzoquinone) and the three roach benzoquinones were calculated with the use of commercially available standards (Fluka Chemie AG., Aldrich Chemical Co., ICN Biochemical Co.). The concentration of internal standard in the extraction solution was chosen to approximate the amount of ethyl-*p*-benzoquinone in the extracts of roach gland(s) or spray (10–45 μg/ml solvent). Serial extractions both of glands and spray-coated vials showed the extraction to be complete after a single extraction step.

Collection of Quinones. Gland contents were obtained either by dissection of the gland or by direct collection of the spray. For gland dissection, weighed and sexed roaches were immobilized by chilling (−9°C for 3–5 min), and glands (with some associated tracheal tissue) were excised with fine stainless-steel forceps. Dissections were performed over KI indicator paper (Eisner, 1958; Roth and Stay, 1958) to provide a visual record of quinone loss (gland rupture; leakage of quinone vapor from spiracular opening) during the dissection. Glands, either singly or as the lumped pair from individuals, were immediately extracted in a weighed amount (to 0.1 mg) of dichloromethane containing the internal standard. Glands were squeezed flat in the solvent with a microcapillary, and aliquots were analyzed within 2 hr of extraction. Values are the means of two to three determinations per sample.

From casual observations of live roaches released on indicator paper, it was clear that the roaches do not discharge their glands spontaneously and that quinones do not leak from the glands in quantities sufficient to register on the indicator paper. However, the roaches discharge in response to attack, which can be simulated by firmly pinching an appendage with a pair of forceps (Eisner, 1958) and in response to CO_2 anesthesia (Roth and Stay, 1958). Both these techniques were used in different experiments in order to quantify the amount of quinone discharged from individual roaches.

Weighed and sexed roaches were coaxed to walk into clean (and in the pinching experiment, chilled) 25-ml scintillation vials and stimulated either by gaseous CO_2 or by pinching of a leg with a pair of forceps. For the anesthesia-induced discharge, a small quantity (approx 30–100 mg) of powdered solid CO_2 (Dry Ice) was placed in the bottom of the vial, the lid was closed, and the vial was rotated so that the roach did not come into direct contact with the Dry Ice. The ice would sublime in less than 40 secs; the roach would convulse (abdominal shuddering), eject a portion of its gland contents, and become immobile. Once immobile (approx. 1 min), the roach was removed, and a weighed amount of dichloromethane containing the internal standard was placed in the vial to extract the ejected quinone. These estimates of the amount discharged should be viewed as minimum estimates due to potential evaporative losses and the possible absorption of benzoquinones to the body of the roach.

Recharge Rates after Molting. We estimated the rate of gland filling in adult roaches after molting by quantifying the quinone contents of roaches at intervals after the last nymphal molt. Freshly molted adult roaches (28 female, 16 male), white in appearance (as they typically are for 2 hr following the molt) were collected from the main colony and placed in 8.5-cm-diam. round containers in groups of no less than 2. All containers were supplied with fresh food and water every three to five days and kept in the laboratory under ambient conditions (20–25°C). At varying times (2 hr to 44 days) after the molt, individual roaches were weighed and sexed and had their glands excised for analysis. Left and right glands were analyzed separately for all roaches in the 2-hr to six-day postmolt category. In all others, left and right glands from individual roaches were combined for analysis.

Recharge Rates after Discharge: Short-Term Experiments. In order to quantify the rates of gland-filling shortly after discharge, we estimated the total quinone production (discharged plus remaining) of roaches induced to spray either three, four, or five times over a 13-day period in the following experiment. Eighteen adult roaches (eight males, 10 females) haphazardly selected from the main colony, were individually confined with food and water. All roaches were labeled, weighed, and on the following days were anesthetized and the quinone discharge quantified: on day 1, all roaches (1–18); on day 3, roaches 1–5; on day 5, roaches 1–10; on days 7 and 12, all roaches (1–18). On day 13, the glands of all roaches were excised for quinone analysis.

Recharge Rates after Discharge: Long-Term Experiments. In order to quantify rates of gland-filling after a single discharge over the long gestation period (85–130 days) of these roaches, we conducted the following experiment. Twenty-nine freshly molted, untanned female roaches with their mates were removed from the main colony and placed as mated pairs in containers with

food and water. In the large stock colony, females mate immediately after molting. In the three cases where the mate was uncoupled in the process of transferring the female from the main colony to the container, another mature adult male was added to the container. The 29 mated pairs were haphazardly assigned to two groups: a control group (10 pairs) and a treatment group (19 pairs) in which the females of each pair were induced to spray once (with CO_2 anesthesia) 15–29 days after molting and mating. Within a 60-hr period following birth, the glands were removed from all female roaches, and their quinone contents were quantified. Gestation time, number of offspring, female weight at molting and parturition, and weight of offspring were also measured.

RESULTS

Gland-Filling after Molt. The observation that the entire gland contents are shed during the molt (Roth and Stay, 1958) was confirmed by chemical analysis. The mean quinone content per gland pair dissected from five last-instar nymphs was 12.4 $\pm$ 4.3 (SE) μg and the amount of quinone present, averaged from two pairs of freshly molted glands from last-instar nymphs, was 19.6 $\pm$ 9.6 μg. Moreover, freshly molted adults have only trace amounts of quinone in their glands (Figure 1).

The glands of freshly molted adults fill at a linear rate (Figure 1 insert) for at least the first six days after molting (μg quinones = 1.4 + 18.1 $\times$ days since molt, $r^2 = 0.86$, $t_{slope} = 11.2$, $P < 0.0001$). The relationship between days since the molt and amount of quinone in the glands becomes more variable 10 days after a molt (Figure 1), and this variability is not appreciably reduced by standardizing for roach mass. Clearly a few roaches were able to fill their glands at the rate established during the first six days after the molt for the 44-day period (Figure 1), but a majority of roaches either decreased or stopped gland-filling (and by inference, quinone synthesis) or, alternatively, discharged their gland contents sometime before dissection and thereby appeared to have attenuated filling rates.

Left and right glands filled in a synchronous manner: a linear regression of left on right gland total quinone contents from 23 roaches was highly significant ($r^2 = 0.95$, $F_{1,21} = 388.1$, $P < 0.0001$) with a slope (1.009 $\pm$ 0.051) not significantly different from unity.

Amount of Quinone Sprayed. Given that in undisturbed roaches, left and right glands contain indistinguishable amounts of quinone, the difference in quinone contents between left and right glands in roaches induced to spray on only one side provides an estimate of the amount sprayed. Utilizing this

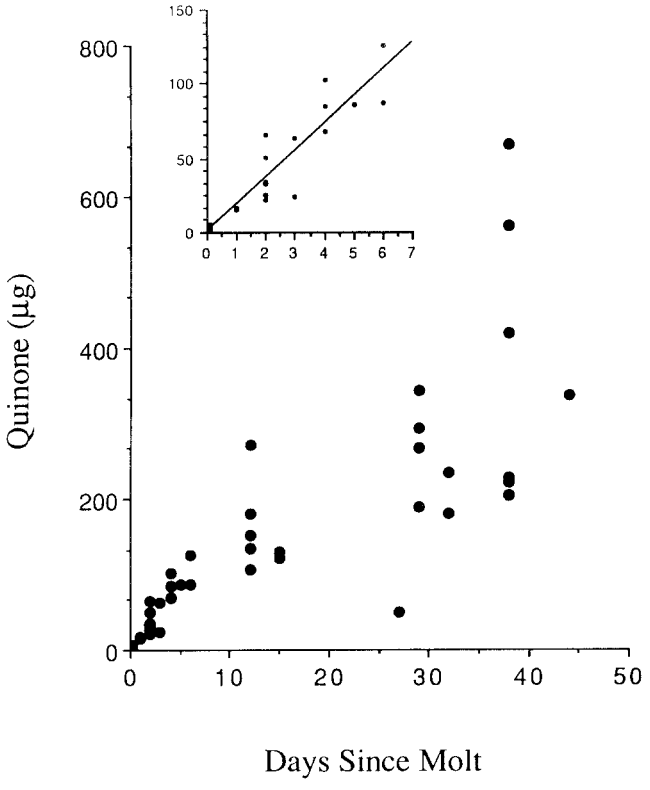


FIG. 1. The glandular quinone content (μg) of 44 adult roaches determined at various times (0.1–44 days) after the last nymphal molt. Insert: A subset of the same data with the best linear fit (μg quinone = 1.4 + 18.8 × days since molt, $r^2 = 0.86$).

approach with four adult roaches induced to spray over indicator paper from only one side, we estimate that in response to a pinch of one leg, roaches can spray highly variable amounts from one gland, ranging from 6 to 173 μg of quinone (Figure 2), which represents 5–77.5% of the total quinone content of the discharged gland.

Roaches anesthetized with CO_2 discharge both of their glands simultaneously. Of the 18 roaches from the short-term recharge experiment, 15 roaches discharged measurable amounts of quinone. The amount sprayed (109.3 ± 21.7 μg) represented 47.9 ± 9.1% of their total quinone contents, which in turn was estimated by summing all discharges plus the amount remaining in the glands at the end of the experiment (Figure 3).

Composition of Secretion. The quinone composition of the secretion

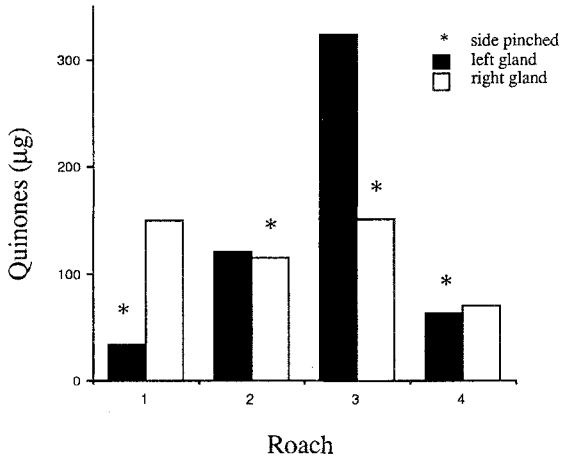


FIG. 2. Quinones (μg) remaining in the left and right glands of four roaches induced to spray on only one side with a firm leg pinch. Glands tend to be depleted on the pinched side of the roaches.

changed in a consistent manner as the glands filled (Figure 4) after the molt. Ethyl-*p*-benzoquinone was the dominant component (70%) shortly after the molt, but its rate of accumulation quickly decreased so that 44 days later it was a lesser component, representing 15% of the total secretion. In contrast, *p*-benzoquinone showed a delayed onset of accumulation but retained a constant rate of accumulation, so that 44 days after the molt *p*-benzoquinone represented 40% of the total secretion. Methyl-*p*-benzoquinone had an intermediate pattern of accumulation; it reached its maximum proportion of the total secretion (60%) at 15 days postmolt, and declined to 45% by 44 days postmolt (Figure 4).

We found that the quinone composition of the spray mirrored that of the secretion in the gland. By comparing the quinone composition of the roaches' largest spray (of the three to five discharges) from the short-term recharge experiment with that of the secretion remaining in the gland, we found no significant differences in the arcsine-transformed percentages of the total quinone content for each of the three quinones (paired *t*s between glands and largest discharge for each roach = 0.10 − 0.28, $df = 16$, Ps > 0.78).

Rates of Recharge: Short-Term Experiment. In contrast with the filling rates of glands measured from 0 to 6 days after molt—estimated as 18.1 ± 1.6 μg quinone per day—the filling rates after discharge were appreciably lower. Roaches induced to spray large amounts had less in their glands at the end of the experiment. For example, had roaches been filling their glands at the postmolt filling rates during the two five-day periods between sprays, individuals in

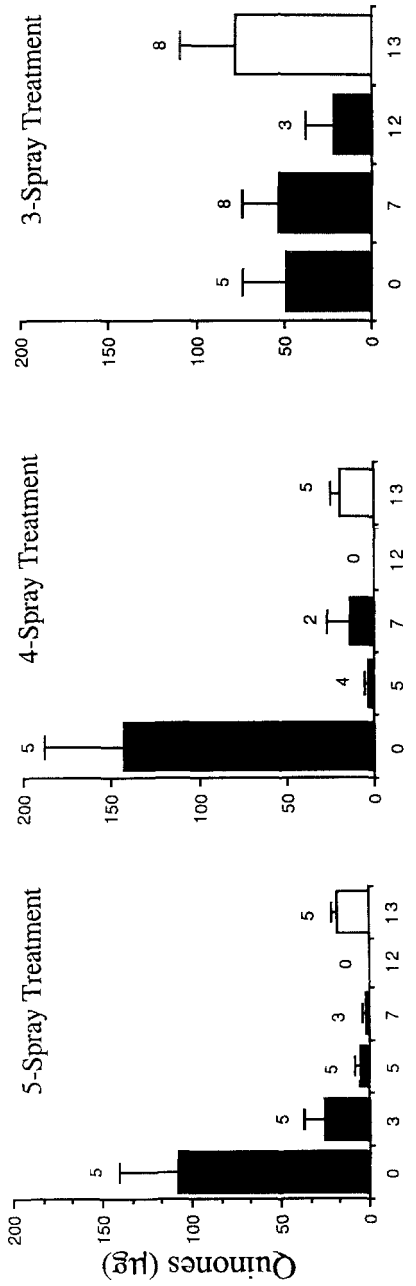


FIG. 3. Quinones (μg) discharged after exposure to Dry Ice (black bars) three, four, and five times over a 12-day period and the amount remaining in the gland (white bars) on day 13. Above the black bars are numbers of roaches discharging a measurable amount of quinone; above the white bars are numbers of roaches in each treatment. All roaches had measurable amounts of quinones in their glands.

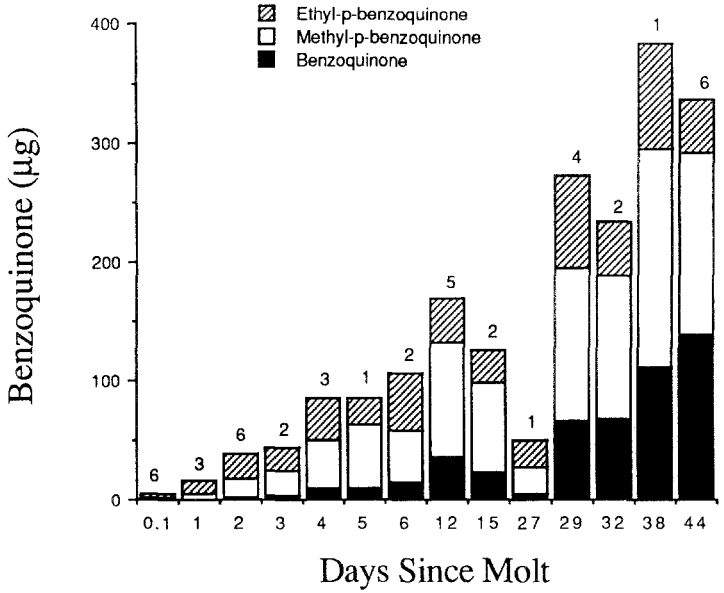


FIG. 4. Quinone composition of the 44 roaches depicted in Figure 1 averaged for each sampling time. Above each column is the number of roaches in each sample. Note that the x axis is nonlinear.

the four-spray treatment could have twice produced 90.5 μg of quinone (5 days $\times$ 18.1 μg/day; Figure 3), which they evidently did not. Moreover, roaches induced to discharge three, four, and five times over a 13-day period did not differ in their total quinone production ($F_{2,15} = 1.53$, $P = 0.248$). Interestingly, the roaches in the four- and five-spray treatments, which discharged no quinone during the final anesthesia, still had, remaining in the gland, a residual amount of quinone (19.8 $\pm$ 4.8 and 17.6 $\pm$ 3.5 μg, respectively) representing 11.2 and 11.0% of the summed total. The roaches in the different spray treatments did not differ significantly in weight ($F_{2,15} = 0.68$, $P = 0.52$).

Rates of Recharge: Long-Term Experiment. The refilling rates of pregnant roaches induced to spray (with CO_2 anesthesia) were similarly low over the long gestation time. Of the 19 CO_2-treated roaches, 18 discharged a measurable amount of quinone (46.27 $\pm$ 13.49 μg). At the end of their pregnancies (85–130 days later), these same roaches had significantly less quinone in their glands than did control roaches not induced to spray ($t = 2.19$, $P = 0.037$) (Figure 5). Total quinone production was not significantly different between the two groups ($t = 1.29$, $P = 0.21$). The glandular quinones appeared to be stable; we saw no evidence of quinone polymerization, even in 130-day-old roaches.

The roaches in the two treatments did not differ (all ts < 0.97, Ps < 0.5)

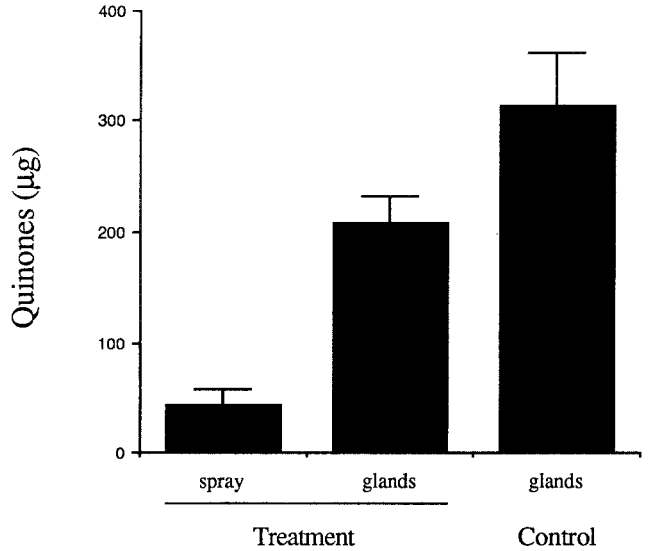


FIG. 5. Quinones (μg) discharged (spray) after an exposure to Dry Ice and in the glands 85–130 days after the discharge in gravid roaches treated (treatment) and not treated (control) with Dry Ice. Gravid roaches induced to spray do not refill their glands even over long time periods.

in weight either immediately after molting and mating (284.1 $\pm$ 6.0 mg) or parturition (266.9 $\pm$ 5.6 mg), and interestingly, they also did not differ in gestation time (121.4 $\pm$ 3.4 days), number of offspring (11.5 $\pm$ 0.2), or the total weight of offspring produced (249.5 $\pm$ 5.7 mg).

DISCUSSION

The adult roaches from this colony had high filling rates shortly after molting. We estimate that lesser amounts of secretion discharged in response to pinching (6 μg of quinone) are therefore replenishable three times a day during early postmolt gland-filling. Thus, even large discharges that depleted the reservoir contents by 50% could be replenished in the course of a week, which is comparable to the filling rates after discharge of the steriod defensive secretions in dytiscid beetles (Fescemyer and Mumma, 1983). Interestingly, approximately 11% of the total gland contents, which represents about one day's worth of quinone accumulation at the high postmolt filling rate, is retained in the gland after repeated stimulation. This quantity of secretion may not be physically sprayable, or the roaches may be withholding this final portion of their secretion for an unknown secondary function.

In contrast to the filling rates observed after molting, the recharge rates observed both over long time periods (85–130 days) in gravid females and short time periods (13 days) in roaches of both sexes were substantially slower. After discharge, roaches did not measurably refill their glands. This observation is subject to two caveats. Roaches induced to discharge their quinones were briefly (2 min) exposed to CO_2 (once in the long-term experiment and up to five times in the short-term experiment). Because carbon dioxide anesthesia is known to have varied and subtle effects on insect metabolism (Nicolas and Sillans, 1989), its effects on quinone metabolism cannot be ruled out. Second, the observed lack of refilling may reflect not a lack of quinone synthesis but merely a lack of accumulation in the gland; after an initial discharge, the glands may become leaky and not refill due to the lack of retention of secretion in the gland. This possibility seems unlikely, given that the gland contents of roaches that had discharged 85–130 days earlier, plus the amount discharged, were equivalent to the gland contents found in undisturbed roaches.

The observation that gland filling appears limited to the early postmolt period is intriguing, because molting and subsequent hardening of the new cuticle require the production of large amounts of quinones for the cross-linking of cuticular proteins during sclerotization and for the production of melanin (Chapman, 1982). This association suggests that the physiological processes associated with ecdysis are also associated with the synthesis of defensive quinones.

Moreover, an examination of the biosynthetic pathways leading to cuticular and defensive quinones suggests that there may be competition for common substrates. Quinones and phenols used in the sclerotization and darkening of insect cuticle are derived from the aromatic amino acid, tyrosine (Chapman, 1982). The beetle *Eleodes longicollis*, which produces the same three quinones in its defensive secretion as *Diploptera punctata*, produces the three structures by two different biosynthetic pathways (Meinwald et al., 1966). The methyl and ethyl derivatives of *p*-benzoquinone are formed from aliphatic acids, while the unsubstituted *p*-benzoquinone is derived from the two aromatic amino acids, tyrosine and phenylalanine. If defensive quinone synthesis in *Diploptera* is similar to that found in *Eleodes*, the delayed onset of accumulation of *p*-benzoquinone compared to the alkylated quinones in the glands after the molt (Figure 4) suggests substrate competition for aromatic amino acids between benzoquinone synthesis in the glands and cuticular quinone synthesis.

Diploptera punctata clearly is able to modulate its defensive discharges and, for a period after molting, is able quickly to fill their gland contents. The apparent lack of refilling after discharge potentially represents an adaptive constraint to the defensive use of this secretion and may be an evolutionary consequence of deriving defensive secretions from an ancestral biochemical pathway that is so important in ecdysis.

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CHEMICAL TAXONOMIC STUDIES OF CUTICULAR HYDROCARBONS IN LOCUSTS OF THE *Schistocerca americana* COMPLEX (ACRIDIDAE: CYRTACANTHACRIDINAE): CHEMICAL RELATIONSHIPS BETWEEN NEW WORLD AND OLD WORLD SPECIES

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Abstract—Genetics studies gave an insight into the evolutionary systematics of the *S. americana* complex. Chemical analysis and comparison of the cuticular hydrocarbons of five species of this complex (*S. gregaria, cancellata, americana, piceifrons*, and *pallens*) confirmed the species status previously established by hybridization and also revealed new elements. We have shown the existence of two groups of species: *S. gregaria* and *S. cancellata* on one hand, *S. americana* and *S. piceifrons* on the other. *S. pallens* does not fit into either of these groups. These analyses thus have shed some light on the possible origins of *S. gregaria* vis-à-vis *S. cancellata* for which an explanation is proposed.

Key Words—*Schistocerca americana* complex, Orthoptera, Acrididae, *S. gregaria, S. cancellata, S. americana, S. piceifrons, S. pallens*, cuticular hydrocarbons, mass spectra.

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INTRODUCTION

The genus *Schistocerca* (Stål, 1873) (subfamily Cyrtacanthacridinae) offers a unique suite of economically important species for taxonomic analysis. The place of the genus in the subfamily is peculiar in that, while the Old World contains a rich radiation of 35 published genera, the New World is represented by only *Schistocerca* species and *Halmenus*, the latter being a clear derivative of *Schistocerca* confined to the Galapagos Islands. *Schistocerca* is the most important locust group economically, its component species causing major crop losses in Africa, the Middle East, and Central and South America. It includes the infamous desert locust *Schistocerca gregaria*, which since 1987 has shown a dangerous population resurgence and even has crossed the Atlantic from West Africa (Ritchie and Pedgley, 1989). This group is represented by a single Old World species *S. gregaria* Forskal, but a rich radiation of New World species. These occupy ecologically richer areas from warm temperate to tropical and subtropical regions. The most controversial group in the genus *Schistocerca* is the *americana* complex. In his revision of the genus, Dirsh (1974) considered that all species formerly included in this complex were subspecies of a single superspecies. More recently in a series of genetic taxonomic studies Harvey (1979, 1981, 1982, 1983) and Jago et al. (1979, 1982) showed that the *americana* complex consists of at least six different species. They were able to analyze the genetic and cytologic interrelationships of the following cultures: (1) *S. americana* (Drury, 1773), Florida stock; (2) *S. americana*, Texas stock; (3) *S. piceifrons* (Walker, 1870), Yucatan peninsula, Mexico; (4) *S. piceifrons*, Peruvian stock; (5) *S. pallens* (Thunberg, 1815), Barbados Island stock; (6) *S. cancellata* (Serville, 1838), Argentinian stock; (7) *S. cancellata*, Chilean stock; and (8) *S. gregaria* (Forskal, 1775), Red Sea stock, Arabian Tahama. From these studies (as summarized by Harvey, 1981), the following conclusions may be deduced:

(1) *S. americana* is confined to temperate United States of America and northeastern Mexico. It shows weak phase-related responses, behaving like a true grasshopper on crowding. The first abdominal segment of the male is not brightly colored at maturity, and the abdominal tergites do not bear conspicuous waxy plates nor is an aromatic secretion produced. Its nearest relative genetically is *S. piceifrons* from Mexico.

(2) *S. piceifrons piceifrons* is distributed in Central America. It shows moderately strong phase-related responses, behaving like a true locust in this respect. The male abdomen becomes yellow at maturity and a strong aromatic secretion is produced by glands within the first abdominal segment. It is conspecific with the Peruvian locust *S. piceifrons peruviana*.

(3) *S. piceifrons peruviana* (Lynch Arribalzaga, 1903) is centered on Peru but in years of major outbreaks extends its range to southern Ecuador, Col-

ombia, and Venezuala, where it may intermingle with the nominate subspecies. Its biological and physiological characteristics resemble the nominate subspecies but shows genetic divergence from it (see Harvey, 1982).

(4) *S. pallens* is distributed from Brazil to Mexico and the southern Caribbean, shows weak phase-related responses, and behaves like a true grasshopper on crowding. The first abdominal segment of the male becomes blue at maturity, but no aromatic secretion or wax platelets are produced. Its closest relative is *S. cancellata*.

(5) *S. cancellata* has its distribution centered in Argentina, with records from Chile, Bolivia, Paraguay, Uruguay, and southern Brazil. It shows very high phase-related sensitivity and is one of the world's most economically important locusts. Like *S. pallens*, the first abdominal segment in the male becomes blue at maturity, but in addition it produces waxy abdominal platelets and copious aromatic secretions from an abdominal gland. It is most closely related to *S. pallens*. Chilean and Argentinian populations are only slightly divergent.

(6) *S. gregaria* has an exclusively Old World distribution. The nominate subspecies *S. gregaria gregaria* is exceptionally phase sensitive, showing an ability to form swarms and larvae bands. Adults migrate great distances. *S. gregaria flaviventris* (Burmeister, 1938) was not examined but it is feebly phase sensitive and is confined to southwestern Africa and the cape. *S. gregaria* lacks blue pigment on the first abdominal segment and does not produce aromatic secretions or waxy platelets on the abdomen at maturity. While the population of Ascension Island resembles *S. cancellata* in its solitariform facies very closely, *S. gregaria* will not successfully cross with *S. americana, S. piceifrons, S. pallens* or *S. cancellata*.

The ecological biology and biogeography of *S. piceifrons* have been reviewed by Harvey (1983) and that of *S. cancellata* and *S. gregaria flaviventris* by Waloff and Pedgley (1986).

Within this framework of systematic and taxonomic results, the chance was taken here to analyze the chemical composition of the cuticular hydrocarbons of the cultures available at the ODNRI to see if chemical analysis would confirm the conclusions delineated above. It is already known that these hydrocarbons show great promise as indicators of conspecificity (Howard and Blomquist, 1982; Lockey, 1980, 1985; Castner and Nation, 1984; Bartelt et al., 1986; Carlson and Service, 1980; Carlson and Bolten, 1984; Lavine et al., 1988; Carlson and Brenner, 1988; Jallon and David, 1987; Jallon and Péchiné, 1989). In a study of the cuticular hydrocarbons of 100 species of *Tenebrionidae*, Lockey (1980, 1985) shows that closely related species have similar hydrocarbons, although in different proportions. For the genus *Schistocerca*, a single comparison of these hydrocarbons was published by Jackson (1982), using previously published results on *S. gregaria* (Lockey, 1976) and *S. vaga* (Nelson

and Sukkestad, 1975) and his own results on *S. americana*. Unfortunately, this comparison led only to fragmentary results. In undertaking a fresh attempt in this field, we were aware of the difficulties involved in a study of closely related species, and we took into account the comprehensive results obtained by Knowles (1987) for *S. gregaria* concerning the variation in the composition of cuticular hydrocarbons with age and sex. By choosing and analyzing these cuticular hydrocarbons for male and female insects (fifth-instar larvae and both immature and mature adults), we hoped to compare *S. americana, S. piceifrons, S. cancellata, S. gregaria*, and *S. pallens*. Details of the origins and laboratory generation used in each case are given below.

METHODS AND MATERIALS

Each of the five species listed above was represented by two samples of different geographical origin, except *S. pallens*. The terms in parentheses appearing below indicate the generation of the insects. For example "(L30)" indicates that the insects were part of the thirtieth laboratory generation reared at the ODNRI (London). The samples were as follows:

1. *S. americana*: (i) Florida, immature and mature adults (L 29), fifth-instar larvae (L 30); (ii) northwest Texas, mature adults (L 30).

2. *S. piceifrons*: (i) subsp. *piceifrons*, Mexico, Yucatan, mature adults (L 29), fifth-instar larvae (L 30); (ii) subsp. *peruviana*, central Peru, mature adults (L 18).

3. *S. cancellata*: (i) Argentina, mature adults (L 47); (ii) Chile, mature adults (L 34), fifth-instar larvae (L 35).

4. *S. gregaria*: (i) Africa, central Sahara [stocks at ODNRI and UPS (Orsay), generation not recorded], fifth-instar larvae, immature and mature adults. (ii) Ascension Island, mature adults (L 5).

5. *S. pallens*: (i) Barbados, mature adults (L 48).

The cultures were reared in 50-liter locust cages as described in Jago et al. (1979, 1982). Cuticular extracts were taken from two or three immature or just sexually mature adult insects (male or female) and three male larvae. The results in the case of *americana* (Florida), *piceifrons* (Yucatan), *cancellata* (Chile), and *gregaria* (Africa) have been derived from two generations. The results based on *S. gregaria* (Africa) form part of a study on this insect developed in collaboration between the laboratories "Chimie Structurale Organique" and "Biologie de l'insecte" at Orsay (Knowles, 1987). The results obtained have been standardized with those of the two samples originating from ODNRI, and it was thus possible to make comparisons with the species from Ascension Island, also cultured at ODNRI.

Each insect was killed by freezing at $-20°C$, then extracted by stirring for 10 min in hexane (5 ml/adult and 3 ml/larva). After evaporation of the

solvent, the hydrocarbons were separated from the weighed extracted cuticular lipids by thin-layer chromatography (Merck 10 × 20 plates of silica gel 60 F254 with concentration zones) and elution with hexane. The branched hydrocarbons were separated from this weighed fraction using 5 Å Linde molecular sieves (as described by O'Connor *et al.*, 1962).

Combined gas chromatography–mass spectrometry (GC-MS) was used to identify the components of the different extracts. Mass spectra were obtained on a Nermag R10-10 spectrometer associated with a PDP8 calculator (Digital Equipment Instrument) and coupled to a Girdel 31 chromatograph with a Ros injector. Fractions were temperature programmed from 200 to 300°C at 3°/min with an isotherm at 300°C, on capillary columns 10 and 25 m long, 0.32 mm wide, coated with CpSil 5 CB Chrompack. The carrier gas was helium at an inlet pressure of 0.25 bars. The fractions were analyzed by GC-MS, either by electronic impact or by positive chemical ionization. In electronic impact, the ionization voltage was 70 eV and the temperature of the ion source was 260°C. In these conditions, generally the ion M-15 is present. Mass scanning was carried out from atomic mass unit (amu) 100 to 600 or 750. Positive chemical ionization was performed using methane generating an internal source pressure of 0.2 torr; the ionization voltage was 90 eV, the temperature of the ion source was 150°, and mass scanning started at 100 amu.

The mass spectra of alkanes were interpreted according to the criteria proposed by McCarthy et al. (1968), Nelson et al. (1972), Nelson (1978), Pomonis et al. (1978, 1980), and Génin et al. (1988). Integration of chromatographic peaks was carried out with a Hewlett Packard integrator coupled to a Varian 3700 chromatograph with a 25-m column as mentioned above, programmed from 40 to 300°C at 4°/min with an isotherm of 30 min at 300°C and a helium flow rate of 18 cm/sec; an on column injector was used.

Retention indices (Ettre, 1964) were calculated with a 25-m column, using the *n*-alkanes from C_{23} to C_{35} identified without ambiguity by GC-MS and present in all hydrocarbonated fractions.

RESULTS

After separation on silica, the cuticular extracts (0.3 mg for the larvae, 3 mg for the mature adults) yielded 50–60% of a mixture of hydrocarbons. In this mixture, analysis by GC-MS showed the presence of five classes of hydrocarbons: (A) *n*-alkanes; (B) terminally branched alkanes; (C–E) internally branched monomethyl-, dimethyl-, and trimethylalkanes, respectively, each chromatographic peak often corresponding to a mixture of isomers.

In Table 1, the *n*-alkanes are expressed as a percentage of the total amount of hydrocarbons while the remaining classes are expressed as a percentage of the total amount of branched hydrocarbons, evaluated after integration of the

GC peaks. The overall variability does not exceed that found between several generations of *Schistocerca gregaria*. The deviation from the mean values is between 2% and 15%, the small deviations being for the highest and narrow peaks and the larger deviations for the small peaks and the broader peaks at the end of the chromatogram. These variations do not interfere with the results enumerated here.

We have presented simple histograms (Figures 1 and 2) rather than the complex chromatograms obtained from the mixtures of cuticular hydrocarbons of each species (The two gas chromatographic traces given in Figure 3 show this complexity). These histograms were derived from the percentages of each branched product as described above, taking into account only the more important branched hydrocarbons (by reason of the mechanism of their biosynthesis: see Blomquist and Jackson, 1979; Lockey, 1980, 1985), i.e., an even number of carbons for the monomethyl- (28C–52C) and trimethylalkanes (34E–52E) and an odd number of carbons for the dimethylalkanes (33D–53D). Thus, the vertical axis represents the percentages of the various branched alkanes, and the peaks are ordered on the horizontal axis according to their retention times (not to scale). The triplets of monomethyl-, dimethyl-, and trimethylalkanes with the same principal chain have been separated out. Each peak on the histogram is labeled by a number, representing the number of carbons, and a letter (C, D, or E) indicating the class of alkane. Thus, 90–95% of the branched hydrocarbons were taken into account despite simplification of the results to highlight important trends.

After analysis of the mass spectra, the preferred positions of the methyl substituents on these hydrocarbons were recorded on Tables 2, 3, and 4 (only for mature adult males). The quantitative results presented are based on fifth-

TABLE 1. COMPOSITION OF CUTICULAR LIPID HYDROCARBONS FOR FOUR SPECIES OF *Schistocerca*[a]

	Schistocerca gregaria		*Schistocerca cancellata*		*Schistocerca americana*		*Schistocerca piceifrons*	
Alkanes	Af	Asc	Arg	Chile	Tex	Fla	Yuc	Peru
n-Alkanes (%T)	52,4	42,5	37,0	32,1	19,3	29,7	28,0	32,7
Monomethylalk. (%R)	35,0	28,5	22,6	27,0	27,6	33,7	25,2	30,2
Dimethylalk. (%R)	57,1	61,6	59,1	56,8	55,6	56,0	70,6	64,6
Trimethylalk. (%R)	7,1	8,6	17,6	15,6	15,4	7,5	4,9	3,5

[a] Each is represented by two samples of different geographic origin: Af = Africa, Asc = Ascension Is., Arg = Argentina, Tex = Texas, Fla = Florida, and Yuc = Yucatan. %T: percentages scaled to 100 of total alkanes. %R: percentages scaled to 100 of branched alkanes (monomethyl-, dimethyl-, trimethylalkanes).

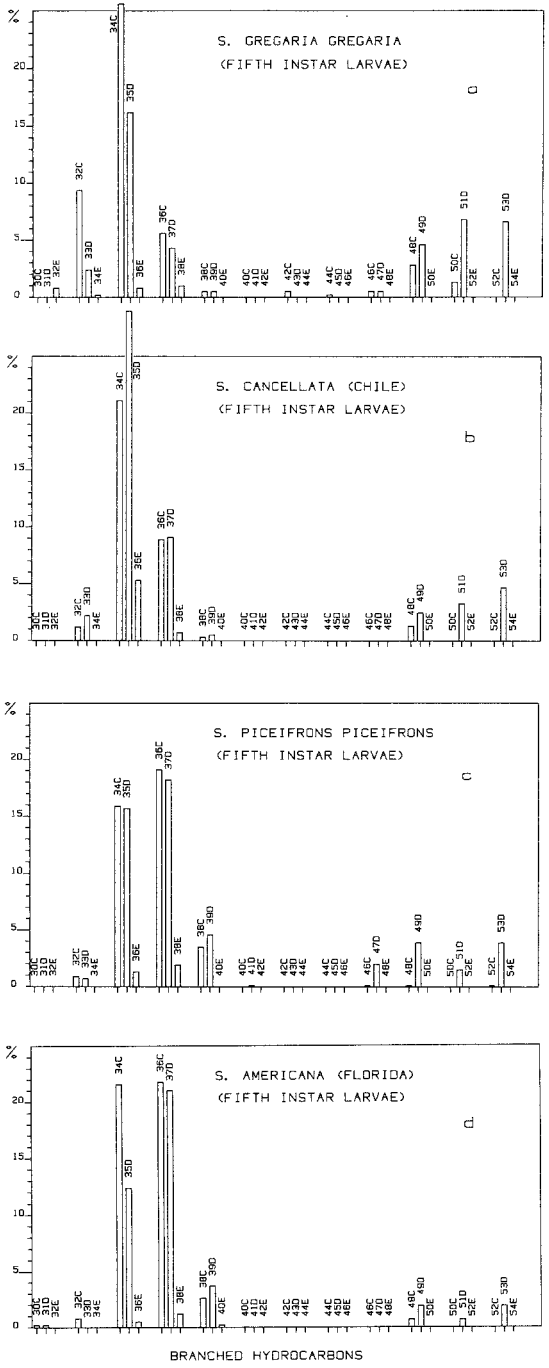


FIG. 1. Percentage composition of major branched hydrocarbons for male fifth-instar larvae of four species of *Schistocerca*.

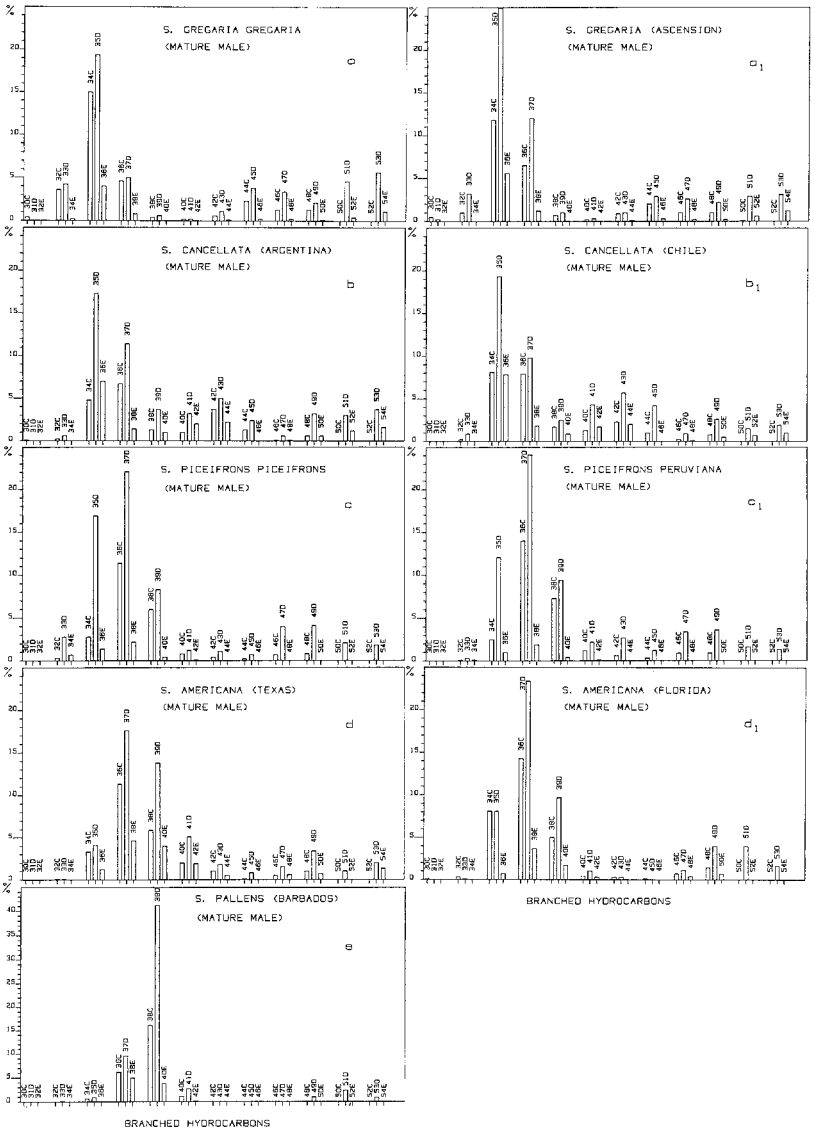


FIG. 2. Percentage composition of major branched hydrocarbons for mature male adults of five species of genus *Schistocerca*. Each species is represented by two samples of different geographical origin, except *S. pallens*: a, a_1; b, b_1; c,c_1; d, d_1; e.

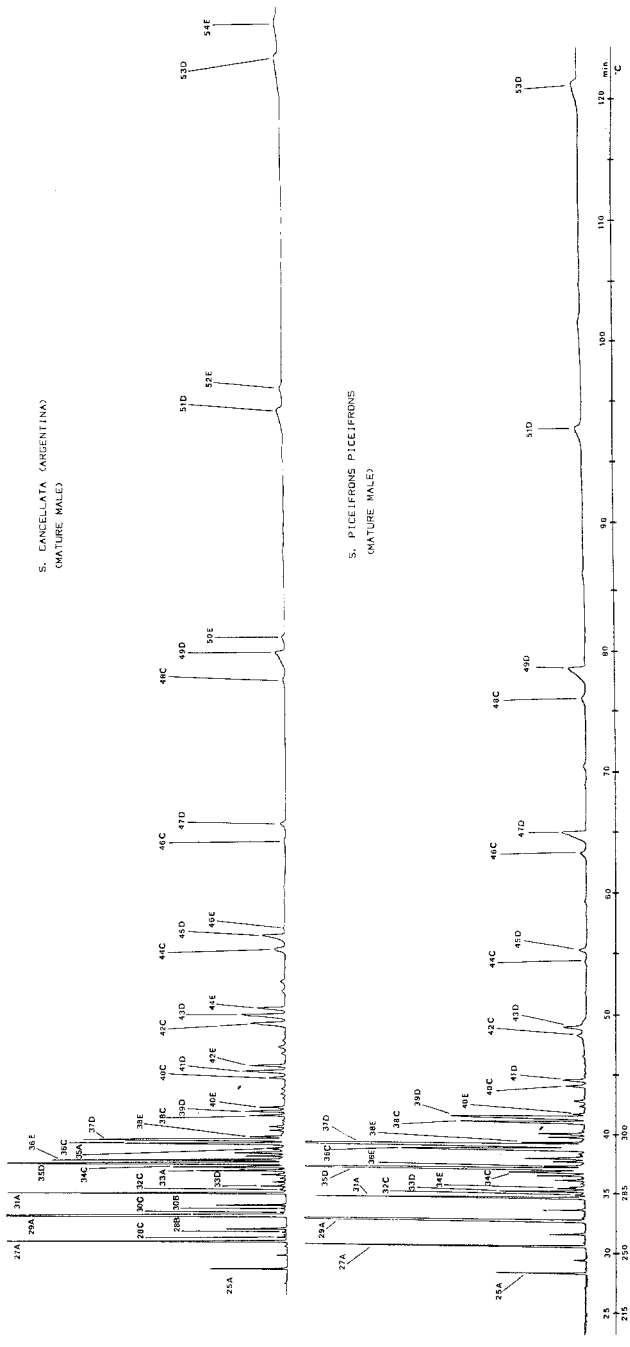


FIG. 3. Gas chromatographic traces of the cuticular hydrocarbons for mature male adults of *S. cancellata* (Argentina), and *S. piceifrons*. Label on the peaks refers to number of carbon atoms and class of the corresponding hydrocarbons.

TABLE 2. MONOMETHYLALKANES: POSITION OF METHYL SUBTITUENTS FOR DIFFERENT ISOMERS IN CUTICULAR HYDROCARBONS OF *S. gregaria, cancellata, americana, piceifons*[a]

Alkanes		*X*-			
X-Monomethyl-	Carbon number; class	*S. gregaria*	*S. cancellata*	*S. americana*	*S. piceifrons*
-Heptacosane	28C	NI	11-*, 13-	9-, 11-, 13-	11-, 13-
-Nonacosane	30C	9-, 11-*, 13-	11-*, 13-, 15-	11-*, 13-, 15-	11-, 13-, 15-
-Hentriacontane	32C	9-, 11-*, 13-	9-, 11-*, 13-	11-, 13-*, 15-	11-, 13-*, 15-
-Tritriacontane	34C	9-, 11-*, 13-, 15-	9-, 11-, I3-*, 15-, 17-	11-, 13-*, 15-*, 17-	11-, 13-, 15-, 17-
-Pentatriacontane	36C	9-, 11-, 13-*, 15-, 17-	9-, 11-, 13-*, 15-, 17-	11-, 13-*, 15-, 17-	11-, 13-*, 15-, 17-
-Heptatriacontane	38C	II-, 13-*, 15-	11-, 13-*, 15-*, 17-	11-, 13-*, 15-, 17-, 19-	11-, 13-*, 15-, 17-, 19-
-Nonatriacontane	40C	11-, 13-*, 15-, 17-, 19-	I3-*, 15-*, 17-, 19-	13-*, 15-, 17-, 19-	13-*, 15-, 17-, 19-
-Hentetracontane	42C	I3-*, 15-, 19-, 21-	13-*, 15-, 17-, 19-, 21-	13-*, 15-, 17-, 19-, 21-	13-*, 15-, 17-, 19-, 21-
-Tritetracontane	44C	13-*, 15-, 19-, 21-	13-*, 15-, 19-, 21-	13-*, 15-, 21-	13-*, 15-, 17-
-Pentatetracontane	46C	13-	13-*, 15-, 21-, 23-	13-*, 15-, 23-	13-*, 15-, 17-
-Heptatetracontane	48C	I3-	13-*, 15-, 21-, 23-	13-*, 15-, 23-	13-*, 15-, 17-, 19-
-Nonatetracontane	50C	13-	NI	NI	NI
-Henpentacontane	52C	NI	NI	NI	NI

[a] Underlining indicates the most abundant hydrocarbons. Asterisks indicate the most important isomers in mixtures.

TABLE 3. DIMETHYLALKANES: POSITION OF METHYL SUBSTITUENTS FOR DIFFERENT ISOMERS IN CUTICULAR HYDROCARBONS OF *S. gregaria, cancellata, americana, piceifons*[a]

Alkanes		*X,Y*-			
X,Y-Dimethyl-	Carbon number; class	*S. gregaria*	*S. cancellata*	*S. americana*	*S. piceifrons*
-Hentriacontane	33D	9,13-*, 11,19-	9,13-	NI	11,15-, 13,17-*, 15,19-
-Tritriacontane	35D	9,13-*, 11,15-, (13,21-)	9,13-*, 11,15-, 13,21-	11,15-, 13,17-*, 15,19-, (13,21-)	11,15-, 13,17-*, 15,19-
-Pentatriacontane	37D	9,13-, 11,15-*, 13,17-	9,13-*, 11,15-, 13,17-, 15,19-	11,15-, 13,17-*, 15,19- (13,21-)	11,15-, 13,17-*, 15,19- (11,19-, 13,21-)
-Heptatriacontane	39D	11,15-*, 13,17-, 15,19-,	11,15-, 13,17-, 15,19-*,	11,15-, 13,17-, 15,19- 13,21-, 13,23-	11,15-, 13,17-, 15,19-, 13,21-, 13,23-
-Nonatriacontane	41D	13,17-, 15,19-*, 17,21- 13,21-	15,19-, 17,21-, 13,21-,	15,19-, 17,21-, 13,21-, 13,23-	13,17-, 15,19-, 17,21- 13,21-, 13,23-
-Hentetracontane	43D	13,21-*, 13,23-	15,19-, 17,21-, 13,21-*, 13,23-	15,19-, 17,21-, 13,21-, 13,23-	13,21-*, 13,23-
-Tritetracontane	45D	13,21-, 13,23-*, 13,25-	13,21-, 13,23-*, 13,25-	13,21-*, 13,23-, 13,25-	13,17-, 13,21-, 13,23-
-Pentatetracontane	47D	13,21-, 13,23-*, 13,25-	13,21-, 13,23-*, 13,25-	13,21-, 13,23-*, 13,25-	13,17-, 13,21-*, 13,23-
-Heptatetracontane	49D	13,21-, 13,23-, 13,25-	13,21-, 13,23-*, 13,25-	13,21-, 13,23-*, 13,25-	13,17-, 13,19-, 13,21-*, 13,23-
-Nonatetracontane	51D	13,21-, 13,23-	13,21-, 13,23-*, 13,25-	13,21-, 13,23-, 13,25-	13,17-, 13,19-, 13,21-*, 13,23-
-Henpentacontane	53D	13,21-, 13,23-	NI	13,21-*, 13,23-	13,21-*, 13,23-

[a] Underlining indicates the most abundant hydrocarbons. Asterisks indicate the most important isomers in mixtures. Parentheses indicate isomers that were detected in small quantities, if at all.

TABLE 4. TRIMETHYLALKANES: POSITION OF METHYL SUBSTITUENTS FOR DIFFERENT ISOMERS IN CUTICULAR HYDROCARBONS OF *S. gregaria, cancellata, americana, piceifons*[a]

Alkanes		*X,Y,Z-*			
X,Y,Z-Trimethyl-	Carbon number; class	*S. gregaria*	*S. cancellata*	*S. americana*	*S. piceifrons*
-Hentriacontane	34E	11,15,19-	NI	11,15,19- (F)[b]	11,15,19-
-Tritriacontane	36E	9,13,21-	9,13,21-	11,15,19-, 13,17,21-*	11,15,19-, 13,17,21-
-Pentatriacontane	38E	11,15,23-	11,15,23-	11,15,23- (*F) 13,17,21- (*T)	11,15,21-, 11,15,19-
-Heptatriacontane	40E	NI	13,19,23-	13,17,25- (*F) 15,19,23-	11,15,21-*, 13,17,23-
-Nonatriacontane	42E	NI	13,19,25-	13,17,25-, 13,19,25-(*F) 15,19,23-	NI
-Hentetracontane	44E	13,21,25-	13,21,25-	NI	NI
-Tritetracontane	46E	13,17,23-, (13,19,*X*-), 13,21,25-	NI	NI	NI
-Pentatetracontane	48E	13,17,23-, (13,19,*X*-) 13,21,25-	13,17,23-, 13,19,23-*, 13,21,25-	13,17,23-	NI
-Heptatetracontane	50E	13,17,23-, (13,19,*X*-) 13,21,25-	13,17,23-, 13,19,23-*, 13,21,25-	13,17,23-, 13,17,25-, (13,19,*X*-)	NI
-Nonatetracontane	52E	13,17,23-, 13,21,25-	NI	NI	NI

[a] Underlining indicates the most abundant hydrocarbons. Asterisks indicate the most important isomers in mixtures. Parentheses indicate isomers that were detected in small quantities, if at all.
[b] F = Florida; T = Texas.

instar larvae (Figure 1) and mature adult males (Figure 2). The corresponding mature adult females also were studied and have the same hydrocarbon mixture as the males, as has already been shown for *Locusta migratoria* (Génin et al., 1986) and for all the stages of *Schistocerca gregaria* (Knowles, 1987). The results take in account only the relative positions of substituents for branched hydrocarbons but not their stereochemistry.

The following commentary presents each class of hydrocarbons. In order to draw attention to the important characteristics of each species, we added Figure 4, which presents four histograms simultaneously for the five species and for each class of hydrocarbons (except class B).

Class A: n-Alkanes. These represent 20–53% of the total hydrocarbons of these insects (Table 1), the American species containing the smallest percentage. They have 23–35 carbon atoms; nonacosane (29A) is the most abundant with an average percentage of 50%, except in *S. americana* (Florida), which, in all the stages studied, contains almost equal proportions of heptacosane (27A) and nonacosane (29A) (30%). These alkanes do not constitute an important element of differentiation for these species as shown in Figure 4a.

Class B: Terminally Branched Monomethylalkanes. 3-Methylheptacosane (28B: about 0.1%), 3-methylnonacosane (30B: less than 0.5%), and 3-methylhentriacontane (32B: about 0.1%) were found to be present in the cuticular hydrocarbons of all these species, as shown for *S. gregaria* (Knowles, 1987) and are not therefore of any use in differentiating them, so they are not shown in Figures 1 and 2. They were not described in preceding papers (Lockey, 1976; Jackson, 1982).

Class C: Internally Branched Monomethylalkanes. Among the species concerned, these represent 22–35% of branched hydrocarbons (Table 1) and constitute an homologous series from 28 to 52 carbon atoms (Table 2, Figures 1 and 2). Their identification by mass spectrometry does not pose any problems. The dominant compounds in this series consist of a mixture of 9-, 11-, 13-, and 15-methyltritriacontanes (34C) with the 11- isomer predominating in *S. gregaria*; the preceding mixture plus a mixture of 9-, 11-, 13-, 15-, and 17-methylpentatriacontanes (36C) for *S. cancellata*, with the 13- isomer predominating; a mixture of 11-, 13-, 15-, and 17-methylpentatriacontanes (36C) for *S. piceifrons* and *S. americana*, also with the 13- isomer predominating. The 9- and 11-isomers seem to be favored in *S. gregaria* and *S. cancellata* for the 28- to 36-carbon molecules, while the position 13- is favored in all species by molecules of 40 or more carbons.

Class D: Dimethylalkanes. These are the most abundant and constitute an homologous series from 33 to 53 carbons, representing 55–70% of branched hydrocarbons in these species (Tables 1 and 3, Figures 1 and 2). They have three, seven, nine, or 11 methylene units between substituted carbon atoms. A study of the substituent positions reveals that the molecules 33D–39D in *S.*

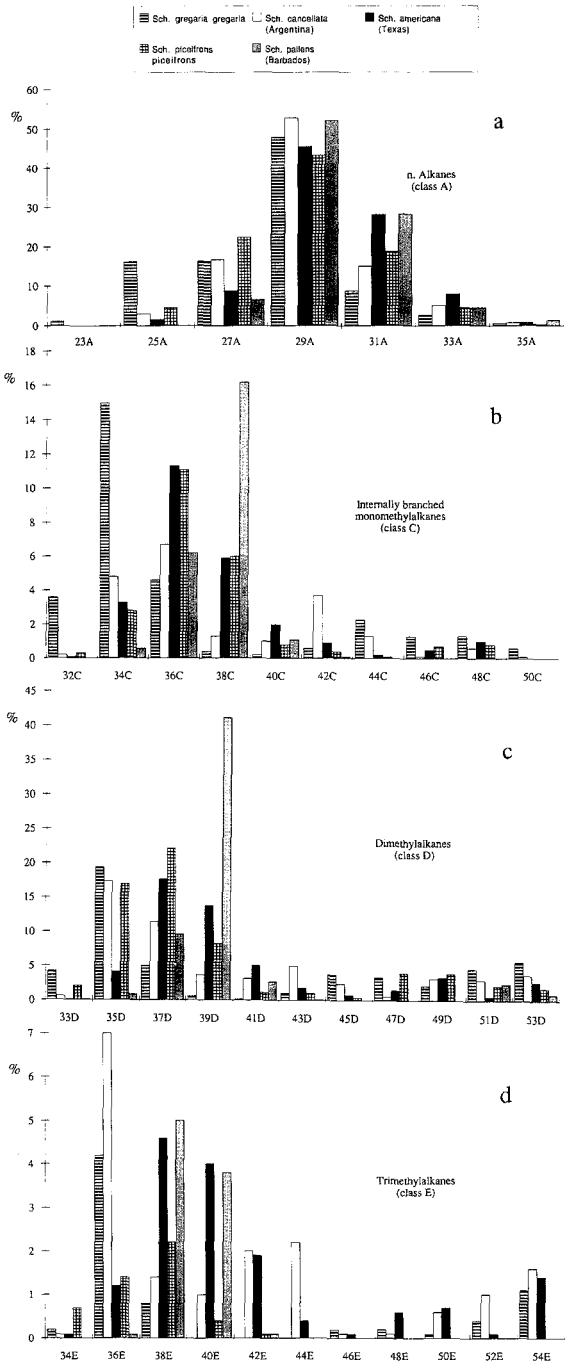


FIG. 4. Percentage composition of major cuticular hydrocarbons for mature male adults of five species of genus *Schistocerca* for each class of hydrocarbons: (a) *n*-alkanes; (b) methylalkanes; (c) dimethylalkanes; (d) trimethylalkanes.

gregaria and *S. cancellata* and 33D–37D in *S. americana* and *S. piceifrons* have, almost exclusively, three methylene units between substituted carbon atoms. The following hydrocarbons are more complex and therefore more difficult to analyze: 39D in *S. americana* and *S. piceifrons*, 41D for the four species, and 43D for *S. cancellata* and *S. gregaria*. These create a bridge between the dominance of molecules with three methylene units separating substituents, and those with a separation of seven, nine, or even 11 methylene units, which are dominant for all species in the hydrocarbons 43D–53D (Table 3).

It is important to note the presence of traces of isomers 13,17- in 45D–51D and 13,19- in 49D–51D for *S. piceifrons*. Traces of the isomer 13,17- for 45D–54D also are observed in *S. americana*, but none of these isomers were found in *S. gregaria* or *S. cancellata*. Nelson and Sukkestad (1975) and Jackson (1982), in studies of *S. vaga* and *S. americana*, respectively, described only those dimethylalkanes with separations of three methylene units between substituted carbon atoms. More recently, however, Nelson et al. (1984) proposed a reinterpretation of three mass spectra that they now attribute to molecules with nine separating methylenes for dimethylheptatriacontane (here 39D) until dimethylhentetracontane (here 43D). This reinterpretation illustrates well the difficulty in interpreting the spectra of these dimethylalkanes; yet it is evident that our results for hydrocarbons 45D–53D in our two *S. americana* samples do not correspond to those obtained by Jackson for the same species. In conclusion, for this class, we note that dimethyltritriacontane (35D) is dominant in both *S. gregaria* and *S. cancellata* with an identical mixture of 9,13- (clearly predominant), 11,15-, and 13,21- isomers, while in *S. americana* and *S. piceifrons* dimethylpentatriacontane (37D) is dominant with a mixture of 11,15-, 13,17-, and 15,19- isomers (the latter two predominating). For these species, Figures 5 and 6 allow a comparison of the spectra of hydrocarbons 35D and 37D for *S. cancellata* and *S. piceifrons* or *S. americana*.

Class E: Trimethylalkanes. Although these represent only 3–17% of the branched hydrocarbons, they play an important role in differentiating the insects (Table 1). They have between 32 and 54 carbon atoms, although not all are present in all the insects studied (Table 4, Figures 2 and 4d). For *S. gregaria* (described by Knowles, 1987) and similarly *S. cancellata*, analysis is simplified by the presence of only one trimethylalkane for the hydrocarbons 34E–44E. For the two other species, analysis was less simple and, for the longer chains (46E–52E), the mixtures were so complex that it was not always possible to fix definitive positions for the substituents. In *S. americana* some identifications (Table 4) were facilitated by the presence, in the two samples, of the same isomers, often in different proportions.

In *S. gregaria* and *S. cancellata*, 9,13,21-trimethyltritriacontane (36E) is dominant, whereas in the other two species 36E is a mixture of the 11,15,19- and 13,17,21- isomers (mass spectra on Figure 7). Similarly, trimethylpentatriacontane (38E), which is dominant in *S. americana* (11,15,23- and 13,17,21-

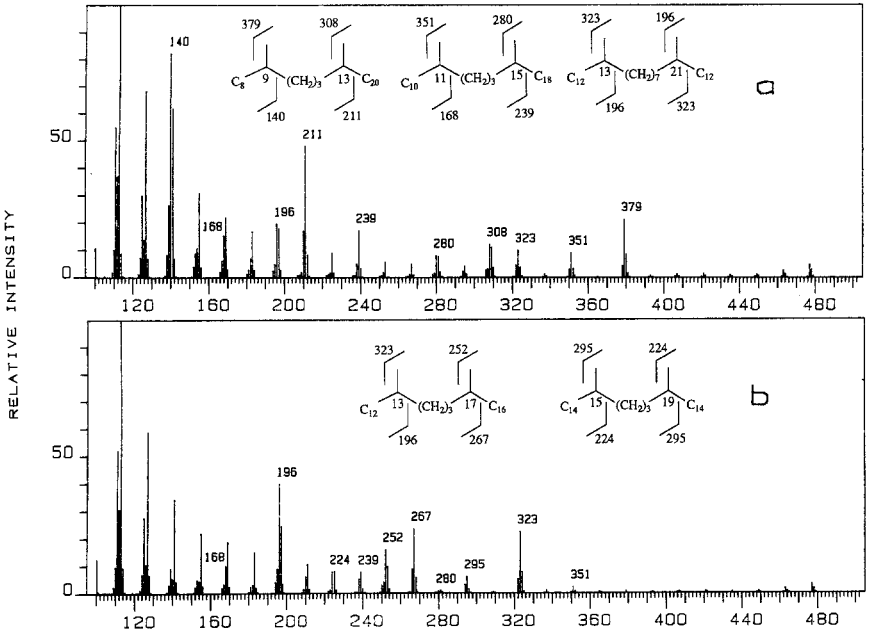


FIG. 5. Mass spectra from dimethyltritriacontanes (35D): (a) *S. cancellata*: 9,13-, 11,15-, and 13,21- isomers; (b) *S. piceifrons*: 11,15-, 13,17-, and 15,19- isomers.

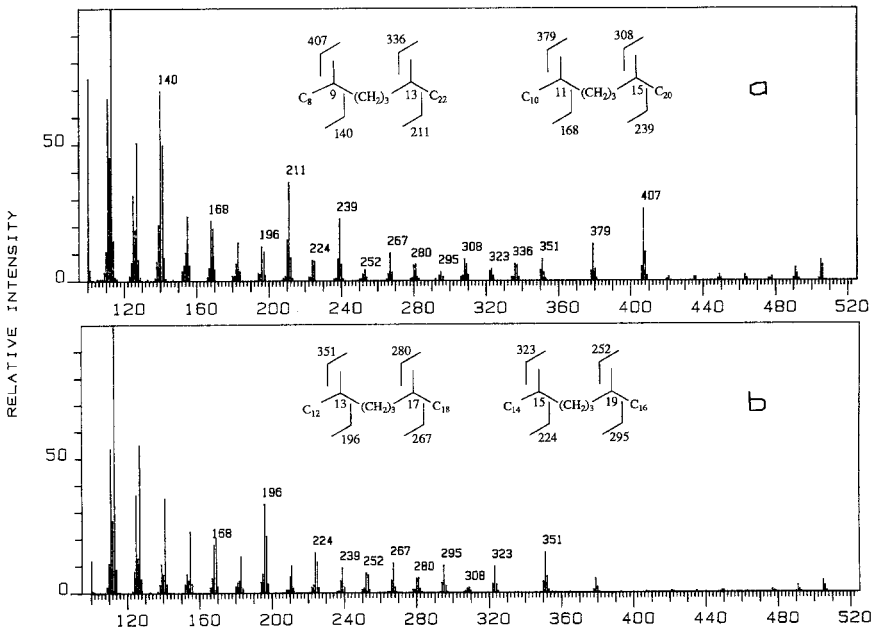


FIG. 6. Mass spectra from dimethylpentatriacontanes (37D): (a) *S. cancellata*: 9,13-, 11,15-, 13,17-, and 15,19- isomers; (b) *S. piceifrons piceifrons*: 11,15-, 13,17-, and 15,19- isomers.

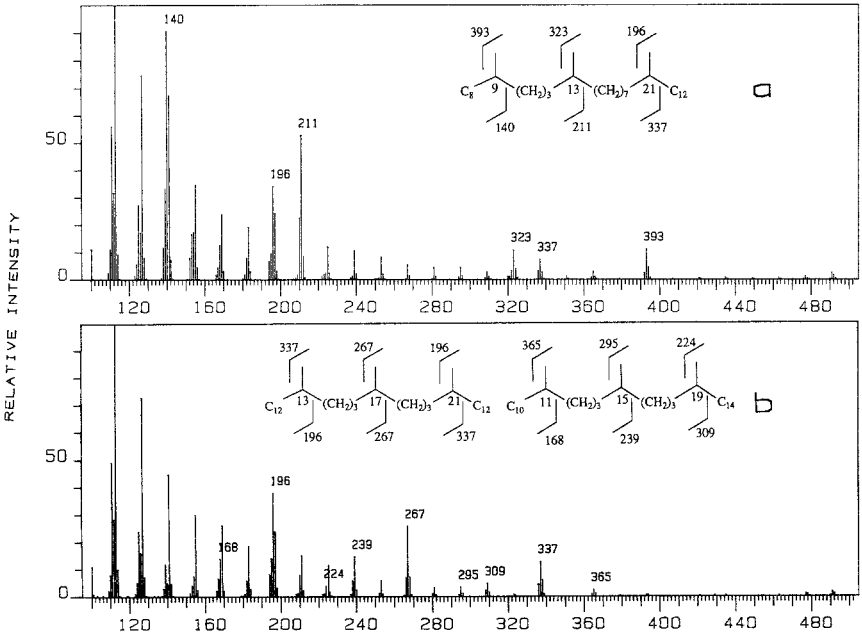


FIG. 7. Mass spectra from trimethyltritriacontanes (36E): (a) *S. cancellata*: 9,13,21-isomer; (b) *S. americana* (Texas): 13,17,21- and 11,15,19- isomers.

isomers) and in *S. piceifrons* (11,15,19- and 11,15,21- isomers), is represented in both other species by the single 11,15,23- isomer (mass spectra on Figure 8). These derivatives have three and three, three and five, or three and seven methylene units between substituted atoms.

DISCUSSION

This study has revealed that, when using cuticular hydrocarbons to determine species relationships, it is necessary to use mature adult insects in order to distinguish closely related species. Thus for the four species studied here, the results of analysis of the cuticular hydrocarbons of fifth-instar larvae showed clearly the separation of the species into two groups (Figure 1, histogram a is very similar to histogram b and the same is true of histograms c and d). However, it was not possible to carry the diagnosis further since the region of peaks 38C–48C, which plays an important role in distinguishing the adult insects (see Figures 2 and 4), is insignificant for the larvae. As has been shown for *S. gregaria* (Knowles, 1987) and *S. americana*, the cuticular hydrocarbons of immature adults resemble, both qualitatively and quantitatively, those of fifth-instar larvae. Thus, for these very near species, we think it is better to discuss the results obtained for mature adult insects.

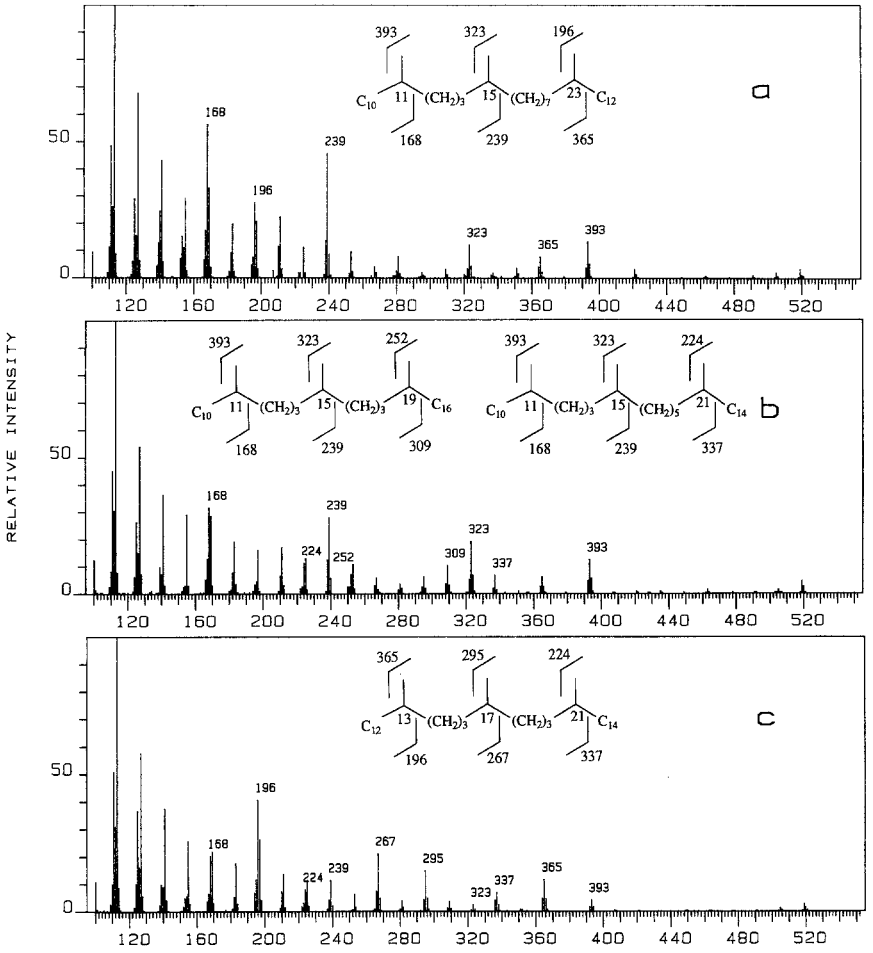


FIG. 8. Mass spectra from trimethylpentatriacontanes (38E): (a) *S. cancellata*: 11,15,23-isomer; (b) *S. piceifrons*: 11,15,19- and 11,15,21- isomers; (c) *S. americana* (Texas): 11,15,23- and 13,17,21- isomers.

It is clear *S. gregaria, S. cancellata, S. americana* and *S. piceifrons* can be classified in two groups of species: *S. gregaria/S. cancellata* on the one hand, and *S. americana/S. piceifrons* on the other. Within each group we find analogies and differences between the species. On comparing chemical composition and histograms a–d with a_1–d_1 in Figure 2, it is clear that the qualitative and quantitative data are almost identical for the pairs of samples of different geographical origin (see later note on *S. americana*). Thus we will compare only the samples for which the data on branched hydrocarbons are given in the first column (Figure 2a–d).

Figure 2a (*S. gregaria*) and 2b (*S. cancellata*) show similarities in their dominant dimethylalkanes, 35D (see also Figure 4c) and trimethylalkanes, 36E (see also Figure 4d), which are in themselves identical with respect to their chemical composition (Tables 3 and 4). Similarly, Figure 2c (*S. piceifrons*) and d (*S. americana*) reveal the dominance of chemically identical methylalkanes, 36C (Table 2 and also Figure 4b), and dimethylalkanes, 37D (Table 3 and Figure 4c), and both have the same dominant trimethylalkane, 38E (Figure 4d) but its composition varies (Table 4). Thus, the dominant branched hydrocarbons (D and E) in *S. americana* and *S. piceifrons* have two carbons more than those dominant in *S. gregaria* and *S. cancellata* as shown in Figure 4.

Although *S. cancellata* and *S. gregaria* have the same dominant hydrocarbons, similar methyl- and dimethylalkanes, and identical trimethylalkanes (whenever these were identified in the two species), they also show notable differences. The most obvious is the increase in trimethylalkanes in *S. cancellata* (double the amount present in *S. gregaria*, Table 1). These results are marked in Figure 2a and 2b, or $2a_1$ and $2b_1$ and in Figure 4d. Histogram 2b also shows that all the branched hydrocarbons from 38C to 44E are present in greater quantities in *S. cancellata*; the tiny quantities of 40E and 42E present in *S. gregaria* were not identified (Table 4). It is quite evident that histograms 2a and 2b correspond to different chromatographic profiles, in agreement with the species status of *S. gregaria* and *S. cancellata* (Jago et al., 1979).

Table 1 indicates a maximum percentage of dimethylalkanes and a minimum percentage of trimethylalkanes for *S. piceifrons* (Yucatan) and the opposite for *S. americana* (Texas). These two species have their dominant hydrocarbons with the same principal chain, 36C, 37D, and 38E (Figure 2c, 2d and Figure 4b–d), but the trimethylalkanes often differ in their chemical composition (Table 4). A comparison of histograms 2c and 2d also reveals that for *S. americana* the peaks corresponding to 38C–44E are more intense, particularly in the case of 40, 42, and 44E (see also Figure 4e); the last two, if they are present in *S. piceifrons*, are in such small quantities that they were not characterized. In *S. piceifrons* the 13,17- isomers of 45D–51D also are seen much more clearly. Thus these two insects, which have related cuticular hydrocarbons, nevertheless present different chromatographic profiles quite in agreement with their status of two different species, as shown by Harvey (1982). In the same work Harvey showed that *S. piceifrons peruviana* and *S. piceifrons piceifrons* were very close genetically and readily hybridized, suggesting a subspecies relationship. The similar histograms 2c and $2c_1$, while not resolving the species–subspecies debate, strongly suggest that they belong to the same species. In contrast, although histograms 2d and $2d_1$ are very close, Table 1 shows a clear decrease in the trimethylalkanes of *S. americana* (Florida) as compared to *S. americana* (Texas), hence the significant lowering of the percentages of the trimethyl peaks in d_1. In fact, all the peaks from 38C to 44E diminish. These two samples of *S. americana* also can be distinguished by their *n*-alkanes (see

Results). [It would seem that *S. americana* (Florida) and *S. americana* (Texas) are chemically divergent in some respects.] Now it is possible to discuss some comments about the very different species. *S. pallens*.

In *S. pallens* (Figure 2e) the same types of hydrocarbons were encountered: *n*-alkanes comprise 21% of the cuticular hydrocarbons, the most abundant being *n*-nonacosane, 29A (52%) and *n*-hentriacontane, 31A (28%); the 3-methylalkanes are represented by 3-methylnonacosane (30B: 0.2%) and 3-methylhentriacontane (32B: 0.2%), but, as in the four other species, these do not constitute a useful distinguishing element (Figure 4a for *n*-alkanes). The number of methylene units between substituted carbon atoms is similar to those of the other species: three, seven, or nine for the dimethylalkanes; three and three, three and five, or five and five for the trimethylalkanes. These identical classes of branched hydrocarbons, and their similar relative proportions (% class D > % class C > % class E >> % class B), would appear to be characteristic of the genus *Schistocerca*.

In *S. Pallens* the preferred sites for the methyl substituents are neither exactly like *S. gregaria* and *S. cancellata* nor like *S. piceifrons* and *S. americana*. They are often close to those described for *S. gregaria* and/or *S. cancellata*: for example, the 9,13-dimethyl isomer is the only isomer for 33D, the most abundant for 35D, and the second most abundant, after the 11,15- isomer, for 37D. The same is true for 36E (9,13,21- isomer) and 38E (11,15,23- isomer). In contrast to the dimethylalkanes, e.g., 39D and 41D, the substituted positions resemble those of *S. americana* and *S. piceifrons*. Similarly, the trimethylalkanes 40E (13,17,25-) and 42E (13,19,25-) are identical to the corresponding favored hydrocarbons of *S. americana* (Florida). The quantitative differences between the branched hydrocarbons are even more apparent (Figures 3e and 4b–d). In fact, 86% of the branched hydrocarbons found in *S. pallens* are composed of 36C, 37D, 38E, 38C, 39D, and 40E. Among these, the most abundant are 38C (16%) and 39D (41%) (Figure 4). It is interesting to note that these hydrocarbons contain four carbon atoms more than the dominant hydrocarbons in *S. gregaria* and *S. cancellata* (34C and 35D) and two more carbon atoms than the dominant hydrocarbons in *S. americana* and *S. piceifrons* (36C and 37D). This increase in chain length also is found for the dominant trimethylalkanes, 38E (5%) and 40E (3.8%).

Thus, an increase in the chain lengths of the dominant hydrocarbons associated with a preference for substitution positions not quite identical to those of the other four species studied are characteristics distinguishing *S. pallens*.

As hoped, analysis of the cuticular hydrocarbons of these five *Schistocerca* species has indicated clear differences (both quantitative and qualitative), in agreement with their species status, a status already established by hybridization studies. The chemical analysis, however, exposes new facts. Indeed, in hybridization between *S. cancellata, S. americana, S. piceifrons*, Harvey concluded

that the results justified the view that these were true species but that the hybridization was rather easier for *S. americana* × *S. piceifrons* than for *S. cancellata* × *S. americana*. After this present study, it is clear that *S. americana* is less closely related to *S. cancellata* than to *S. piceifrons*. Moreover, the chemical resemblances of *S. gregaria* and *S. cancellata* is a good indication of common ancestry for these two species, which now live in the Old World and New World, respectively. The cuticular chemistry also suggests that *S. piceifrons* and *S. americana* originate from a common ancestor, rather than from a *S. cancellata* ancestral line, despite the fact that the latter and *S. piceifrons* are now swarming species with similar phase-related behavior.

The present study revealed that cuticular hydrocarbons of the larvae of *S. cancellata* and *S. gregaria* are virtually identical, whereas that of the adults is related, but its elements are quantitatively different. The origins of the *Schistocerca americana* complex are still a mystery. However, if generic richness is an indication, the subfamily Cyrtacanthacridinae must have its origin in the Old World. A chance for invasion of the New World by proto-*Schistocerca* would have occurred 100–110 million years BP as the continent separated. Following radiation and species enrichment, the proto-*gregaria* element could have returned during the period 70,000–15,000 years BP, when more powerful wind systems would have made passage from Argentina to southwest Africa easy compared to the present day (Van Zinderen Bakker, 1975). This would give us the reason for the close affinity of *S. cancellata* with *S. gregaria*. At 70,000 years BP, aridity extended from southwest Africa to the Congo basin and an arid corridor extended between 25,000 and 12,000 years BP between southwest Africa and Somalia (Hamilton, 1976); Winterbottom, 1967). *S. gregaria* would have been able to expand along this corridor to occupy the less arid Baharo-Eremian belt (30,000– 20,000 years BP). Temperate conditions extended as far south as the Ahaggar (Moreau, 1966), and Van Zinderen Bakker (1969) considers that glacial cooling in northern latitudes led to grasslands spanning the Sahara at this period. Later, between 12,000 and 3000 years BP, a tropical wind system prevailed, promoting evolution of long-distance dispersal in *S. gregaria gregaria* and leaving *S. gregaria flaviventris* as a comparatively non-phase-sensitive subspecies in southwest Africa.

This chemical study is the latest in a multidisciplinary series of taxonomic investigations of the genus *Schistocerca* and must be considered preliminary. An examination of *S. pallens*, for example, which on morphological and genetic ground has been linked closely with *cancellata* (Harvey preliminary study, unpublished), showed that its cuticular chemistry is quite different from that of *S. cancellata, S. gregaria, S. piceifrons*, or *S. americana*. It is hoped that this paper will encourage other scientists to investigate this remarkable genus more fully from the evolutionary point of view, not only for its intrinsic biological interest, but also because of its economic importance.

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CHEMOATTRACTION IN *Pellioditis pellio* (NEMATODA: SECERNENTIA) IN THE PRESENCE AND ABSENCE OF BARRIERS

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Abstract—*Pellioditis pellio* (Nematoda) adults showed heterosexual chemoattraction and female homosexual chemoattraction in an in vitro bioassay without barriers. Male-to-female heterosexual, but no homosexual chemoattraction, was observed when M_r 25,000 pore size dialysis tube chimneys (barriers) were used to restrain female attractor worms. Less attraction was seen when M_r 12,000 pore size barriers were used. The results suggest the presence of at least two types of chemoattractant for this species and that at least one heterosexual chemoattractant is between M_r 12,000 and 25,000.

Key Words—*Pellioditis pellio*, *Rhabditis pellio*, Nematoda, heterosexual chemoattraction, homosexual chemoattraction, pheromone, behavior.

INTRODUCTION

Because of its availability and ease of cultivation, *Pellioditis pellio* (synonym of *Rhabditis pellio*) is an excellent potential model for chemoattraction studies. The only two chemoattraction studies reported for this species found heterosexual, but not homosexual, attraction of adult male and female worms using a nonbarrier in vitro bioassay design (Somers et al., 1977a, b). However, the

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studies had several important limitations, including their unexplained reference to "nematode equivalents," the use of flavobacterium "lawn" in their bioassays, which may have resulted in intrinsic chemical signals that could confound those of the nematodes, and the use of multiple responders in each experiment, which could result in both uncontrolled chemotactic and thigmotactic responses.

We recently initiated studies on the cultivation, biology, and SEM topography of *P. pellio* (Eveland et al., 1990), with the goal of using this nematode in chemoattraction studies. In this, our first chemoattraction study with this species, we describe behavior in barrier and nonbarrier bioassays modeled after previous work with *Schistosoma mansoni* (Eveland et al., 1982; Eveland and Haseeb, 1986), which has the advantage of single male and female worms migrating on an agar substratum essentially free from bacteria.

METHODS AND MATERIALS

As described previously (Eveland et al., 1990), virgin male and female adult *Pellioditis pellio* (formerly *Rhabditis pellio*) were cultured following their isolation from the lumbricoid oligocaete *Apporectodea caliginosa* recovered from soil in Long Beach, California, U.S.A. Chemoattraction studies were done with 3- to 7-day-old worms, using both nonbarrier and barrier bioassays, as no differences were detected in worms of different ages.

The bioassays were modifications of designs used in chemoattraction studies on trematodes. Briefly, the nonbarrier bioassays consisted of polycarbonate chambers as described by Eveland et al. (1982) and Eveland and Haseeb (1986), with a 1% agar substratum but without the Earle's medium overlay, which is necessary for trematodes but not for nematodes. Male–female, male–male, or female–female worm combinations were placed 5 mm apart in separate channels (this distance allowed the worms to move in either direction, and the distance between worms was measured at 5-min intervals for 30 min). Negative experiments were extended to at least 1 hr. Worm migration was studied using a closed-circuit video system, which consisted of an RCA black and white 1-in. Cohu Low Light camera No. 4410 with a 25-mm f1.4 lens, a 20-mm extension tube, and a Panasonic NV8200 video cassette recorder. By trial and error we found that best results were obtained in the nonbarrier design when worms were initially placed approximately 5 mm from each other and in the barrier design when chimneys were placed 5 mm from each other with the responder worm equidistant between the two chimneys. Distances between worms were scored as percent attraction as determined by the formula $(O - D/O) \times 100$, where O is the original distance between worms and D is the distance at a particular time point. Kruskal-Wallis one-way analysis of variance (ANOVA) was used to test differences among the worm combinations at each time point.

In the barrier design, which was a modification of that described by Imperia et al. (1980), dialysis tube chimneys with approximately 12,000 and 25,000 molecular weight (M_r) exclusion sizes (VWR Scientific, Plainfield, New Jersey) were placed 5 mm apart in the chambers; a worm was placed in one of the chimneys and a second worm of the same or opposite sex was placed equidistant between the occupied and unoccupied chimneys. Time periods, calculations, and statistical analysis were the same as for the nonbarrier design.

RESULTS

In the nonbarrier design (Figure 1), the greatest attraction was seen between male and female worms. Although no attraction was apparent between male worms, homosexual attraction between females was observed. Male-to-female attraction differed significantly from male-to-male attraction at 25 and 30 min ($P = 0.039$; $P = 0.050$), and female-to-female attraction differed from male-to-male attraction after 30 min ($P = 0.009$), but male-to-female was not significantly different from female-to-female attraction ($P = 0.923$).

In barrier designs (Figure 2), no difference was seen between random migration of males or females after 30 min ($P = 0.157$). When random migration of males was compared to attraction of males to females inside dialysis tube chimneys with M_r 12,000, the differences were significant after 30 min ($P = 0.047$), although the attraction was much greater when M_r 25,000 was used.

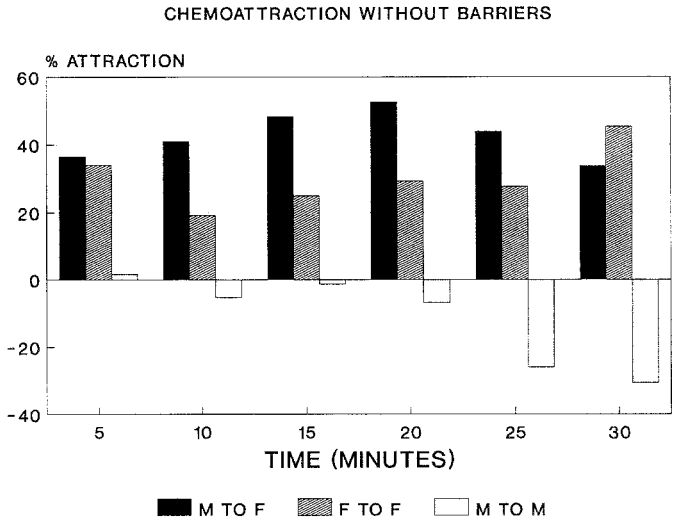


FIG. 1. Summary of 45 experiments without barriers using 14–16 randomly selected male and female combinations of *Pellioditis pellio*.

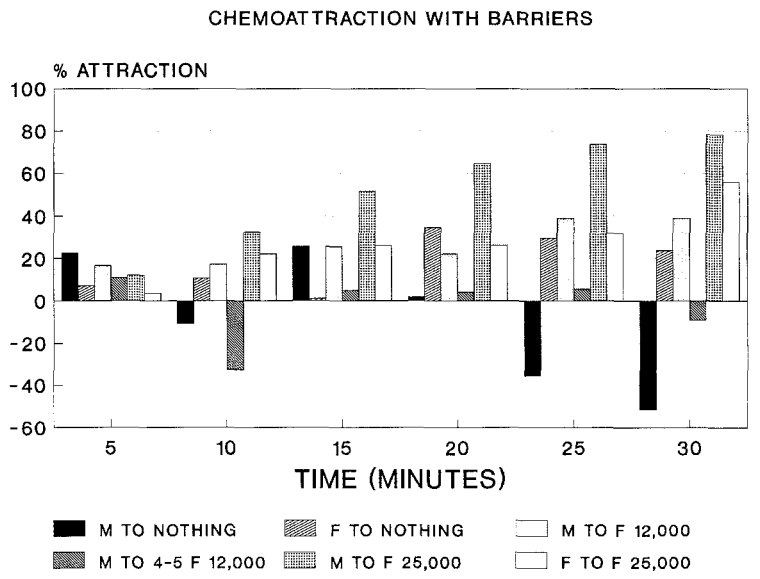


FIG. 2. Summary of 39 experiments with barriers using five to nine randomly selected male and female combinations of *Pellioditis pellio*.

Increasing the number of females inside chimneys to four or five did not cause significant attraction ($P = 0.478$). Migration of males to females inside chimneys with M_r 25,000 differed significantly with random migration of males after 30 min ($P = 0.033$). Females did not appear to be attracted to males, even when chimneys of M_r 25,000 were used. When compared to random migration of females, no difference was noted ($P = 0.167$). There appeared to be slight attraction between females when M_r 25,000 chimneys were used, but the difference was not statistically significant when compared to random female migration ($P = 0.625$). Although negative experiments were extended to at least 1 hr, no further attraction was observed beyond 30 min.

DISCUSSION

Our heterosexual nonbarrier attraction findings that *Pellioditis pellio* males and females attract each other are in agreement with the findings of Somers et al. (1977b), and both males and females also were attracted to the opposite sex in our barrier bioassay. However, we also found evidence for female homosexual attraction in our nonbarrier design, while Somers et al. (1977b) reported a tendency for same-sex worms to repel each other.

Direct comparisons are difficult, however, because the bioassay used by

them utilized multiple responders rather than the one-on-one design used in our study. When multiple responders are used, there are almost certainly pheromonal and tactile interactions between them that may affect their migration to an attractor of the opposite sex. In previous studies with schistosomes, we observed that worm pairs, as well as two or more worms of one sex failed to attract or repel worms of the opposite sex (Imperia et al., 1980; Eveland et al., 1983).

The observation that heterosexual male-to-female attraction appeared strongest in chimneys of M_r 25,000 pore size suggests the possibility that the size of at least one of the chemoattractants was between M_r 12,000 and M_r 25,000. Another possibility, that the larger pore size allowed more rapid diffusion of the chemoattractant through the barrier, enabling the effect to be seen sooner, is unlikely because we extended all negative experiments for at least 1 hr without observing attraction. Furthermore, when it occurred, attraction was usually seen within 5–15 min, and it did not increase even after 1 hr when four or five females were inside the smaller pore size chimneys.

Finding significant female homosexual attraction in the absence, but not in the presence, of barriers also suggests the possibility that more than one type of chemoattractant exists for this species.

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CASTE-DEPENDENT REACTIONS TO SOLDIER DEFENSIVE SECRETION AND CHIRAL ALARM/RECRUITMENT PHEROMONE IN *Nasutitermes princeps*

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Abstract—The soldier frontal gland secretion of *Nasutitermes princeps* induces strong short-range caste-specific alarm and attraction in both soldiers and workers. Soldiers are excited and patrol the surroundings of the source. The secretion per se does not induce ejection of additional secretion. Large workers of the second stage or older are massively attracted when tested in homogeneous groups. They focus their activities much more accurately than the soldiers around the source. The workers' reaction is less intense in the presence of soldiers. Large and small workers of stage 1 scarcely react at all to the secretion, whether tested in homogeneous or mixed groups. These results suggest the following complementary roles of soldiers and workers in defense. The first line of defense is provided by soldiers, which immobilize and incapacitate mobile enemies with their sticky secretion. Defense then is completed by older large workers as they eliminate the source of disturbance. The absence of reaction of young workers, small or large, confirms previous reports on age polyethism in *Nasutitermes* observed in other contexts: young workers tend to stay in the nest. Alarm reactions are elicited by a source of (+)-α-pinene, the major monoterpene in the secretion, while its enantiomer, almost absent from the secretion, induces a much weaker reaction.

Key Words—*Nasutitermes princeps*, Isoptera, Termitidae, termites, defense, alarm recruitment, pheromone, α-pinene, chirality, polyethism.

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INTRODUCTION

In many Nasutitermitinae, the soldiers are able to squirt a glue onto their enemies through their elongated rostrum. This defensive secretion, produced by the soldiers' frontal gland usually consists of a mixture of mono- and diterpenes (Prestwich, 1979, 1983), although sesquiterpenes are also present in some species (Prestwich and Collins, 1981; Valterova et al., 1987, 1988; Everaerts et al., in preparation). Monoterpenes are toxic to insects by topical application and vapor exposure (Everaerts et al., 1988a, and references therein). They are also irritant and may lead the termites' enemy to further entangle itself by scratching the sprayed area. Moreover, they reduce the evaporation rate of the monoterpenes, prolonging their action (Eisner et al., 1976).

Besides its purely defensive role, the frontal gland secretion has in some instances been shown to elicit alarm among the members of the colony. For example, if a squashed soldier head is placed in the vicinity of a foraging column of *Hospitalitermes monoceros*, most termites turn back and run away (Maschwitz and Mühlenberg, 1972). According to Vrkoc et al. (1978), the introduction of various monoterpene vapors in cultures of *Nasutitermes costalis* and *N. rippertii* induces the release of additional frontal gland secretion by the soldiers. Various alarm reactions triggered by soldier defensive secretion also have been reported in other nasute termites (Ernst, 1959; Moore, 1974; Eisner et al., 1976; Stuart, 1981; Mill, 1983), or in termites belonging to other subfamilies (Moore, 1968). However, the frontal gland secretion does not elicit alarm reaction in *Nasutitermes ephratae* (Maschwitz, 1966).

The frontal gland secretion of *N. princeps* (Desneux), a common species in northeastern New Guinea, has been extensively studied (Braekman et al., 1980; Dupont et al., 1981; Roisin et al., 1987; Everaerts et al., 1988b, 1990). The frontal secretion of *Nasutitermes princeps* consists of 89% diterpenes and 11% monoterpenes. (+)-α-Pinene accounts for more than 80% of the monoterpenic content, according to the samples, and its optical purity reaches 99.5% (Everaerts et al., 1990), as recently observed by Lindström et al. (personal communication) in *N. ephratae* and *N. nigriceps*.

In this paper, we will focus our attention on the behavioral effects of the frontal gland secretion on the various castes of *N. princeps*, and, in addition, we will examine the pheromonal role of (+)-α-pinene compared to its enantiomer, absent from the natural secretion.

METHODS AND MATERIALS

Materials. Arboreal nests of *N. princeps* were collected around Hansa Bay, Bogia District, Papua New Guinea. Termites to be used in bioassays were kept in large nest pieces on a water-trapped table and fed with pieces of wood. They

remained active and in good condition for at least one week, after which they were discarded and replaced by new ones.

The standard monoterpenes were commercially available compounds (Fluka AG); their GC purity was above 99%. The optical purity of (+)- and (−)-α-pinene were, respectively, 92% ($[\alpha]_D^{20} = 48°$) and 85% ($[\alpha]_D^{20} = 45°$), based on the value reported by Comyns and Lucas (1957).

Methods. The experimental setup is shown in Figure 1. Groups of 40 individuals selected according to their caste and age (see Results) were put in 9-cm Petri dishes, whose bottom was covered with filter paper. On one side of the dish, a glass tube filled with water and plugged with hydrophilous cotton provided moisture, and a small piece of wood provided food and a substrate on or around which the termites invariably gathered (Figure 1A). A circle (33 mm diameter) was drawn with a soft pencil on the filter paper at the opposite side of the dish. A small hole was drilled in the top of the Petri dish, above the center of the circle, allowing the introduction of the item to be tested, i.e., either a paper impregnated with natural secretion or a droplet of hexane solution of monoterpenes (1% v/v). The termites were left undisturbed for a few hours before being tested.

Natural secretions were obtained by squeezing soldier heads with forceps. The droplet of secretion oozing at the nasus tip was collected on a small square (4 mm^2) of filter paper. Once three soldiers had been squeezed, another square

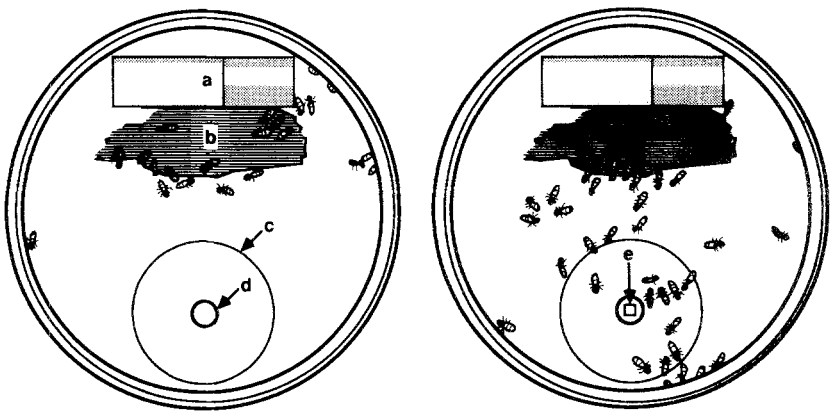


FIG. 1. Attraction of large workers (LW2+) by soldier secretion (after photographs). (A) Situation observed before the introduction of the secretion [a: glass tube filled with water providing moisture, b: piece of wood, c: circle (33 mm diameter), d: small hole drilled in the top of the Petri dish]. (B) Situation 35 sec after the introduction of a piece of filter paper impregnated with the secretion (e).

of filter paper was used to cover the secretion and so prevent it from sticking accidentally to a termite leg and being dragged.

A Hamilton microsyringe was used to introduce 0.5 μl of either bidistilled hexane as a blank, or 1% hexane solution of (+)- or (−)-α-pinene. In *N. princeps*, 0.005 μl (the test amount) is approximately the average amount of (+)-α-pinene contained in three soldier heads.

The number of individuals inside the circle was noted every 30 sec or every minute for 3 to 5 min after introduction of the tested paper or droplet of solution.

Most experiments were binary comparisons: 20 experimental Petri dishes were prepared, of which 10 would receive one treatment and 10 another. All dishes were treated in short succession, and the same treatment was never repeated more than four times in a row, to ensure a sufficient alternation. This procedure was adopted in order to apply both treatments under similar conditions and so obtain two independent series of results suitable for comparison. Indeed, the reactions of the termites showed quantitative but not qualitative variations when tests were performed with insects from different nests, at different times. In one experiment, described in Figure 4 below, three treatments were compared following the same principle. All graphs show the average numbers, with standard error bars, of individuals inside the experimental circle during the course of the bioassays. After each experiment, the termites, pieces of wood, and filter papers were discarded and the glass tubes and the Petri dishes were cleaned with alcohol.

Four categories of individuals were tested: soldiers, large (female) workers of the first instar (LW1), large workers of the second instar or older (LW2+), and small (male) workers of the first instar (SW1). Older small workers are very scarce in *N. princeps* (see Roisin and Pasteels, 1986, for a detailed description of polymorphism and postembryonic development in this species).

RESULTS

Response of Various Castes of N. princeps to Soldier Secretion. The introduction of a virgin paper never elicits any reaction. When tested alone, older large workers quickly rush toward a filter paper impregnated with soldier secretion (Figure 1B, see also Figure 4b below). The reaction reaches its maximum after 60–90 sec and then gradually subsides. Comparative tests with mixed groups of workers of different subcastes show that LW2+ react much more markedly than LW1 (Figure 2) and SW1 (Figure 3). Mixed groups of young workers (LW1 and SW1) hardly display any reaction at all.

Figure 4 presents the results of comparative tests between LW2+ and soldiers in either homogeneous or mixed groups. In homogeneous groups, LW2+ proceed en masse toward the soldier secretion (Figure 4b). Pure groups of sol-

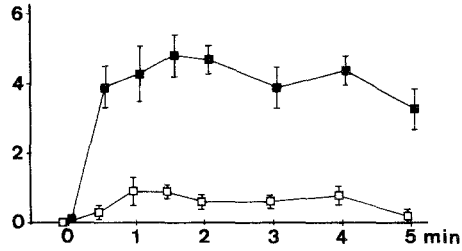


FIG. 2. Comparative attractiveness of soldier secretion to large workers of different stages: number of workers around the source over time. Mixed groups of 20 large workers of stage 2 or older (LW2+, ■) and 20 large workers of stage 1 (LW1, □). Mann-Whitney U test: $P < 0.001$ (0.5–4 min), $P < 0.01$ (5 min).

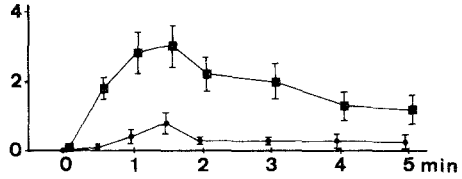


FIG. 3. Comparative attraction of large and small workers to soldier secretion: number of workers around the source over time. Mixed groups of 20 LW2+ (■) and 20 small workers of stage 1 (SW1, ●). Mann-Whitney U test: $P < 0.001$ (0.5–1.5 min), $P < 0.01$ (2 min), $P < 0.05$ (3–5 min).

diers display excitement and attraction, yet their reaction is less precisely focused on the test paper, and relatively few individuals remain within the experimental circle (Figure 4a). The soldiers were never observed to squirt their secretion during the tests. Interestingly, the reaction of LW2+ in mixed groups of workers and soldiers (Figure 4c) is much weaker than in pure worker groups (Figure 4b).

Response of N. princeps LW2+ to (+)-and (−)-α-Pinene. The massive reaction of LW2+ was used to quantify the response to solutions of chiral α-pinene.

Pure *n*-hexane elicits some reaction in *N. princeps* workers. However, the addition of 1% (+)-α-pinene greatly increases their response. The maximum response is reached sooner (30 sec) and the reaction fades out faster that with natural secretion (Figure 5). (+)-α-Pinene is more active than its enantiomer, (−)-α-pinene (Figure 6). Note that in our assays, the reaction to (−)-α-pinene might even have been enhanced by impurities of (+)-α-pinene, since the optical purity of the available (−)-α-pinene was only 85%.

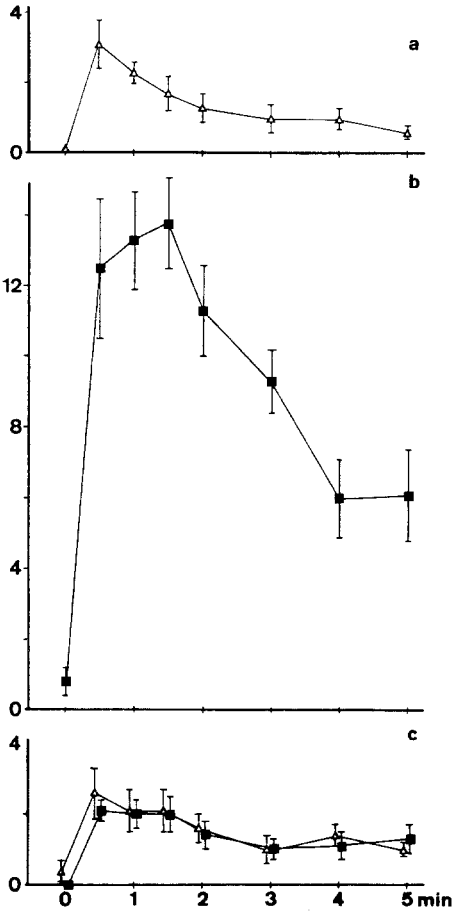


FIG. 4. Comparative attractiveness of soldier secretion to soldiers (△) and large workers of stage 2 or older (LW2+, ■): number of individuals around the source over time. (a) Homogeneous group of 40 soldiers. (b) Homogeneous group of 40 LW2+. (c) Mixed group of 20 LW2+ and 20 soldiers. Mann-Whitney U test: Soldiers (a vs c) NS; workers (b vs c) $P < 0.01$ (0.5 min), $P < 0.001$ (1–3 min), $P < 0.05$ (4–5 min).

DISCUSSION

An alarm effect has been assigned to the frontal gland secretion of several termite species. However, the so-called "alarm" behaviors vary widely according to the species. These various reactions could depend on the context in which the behaviors were observed and also on interspecific differences. For instance, in *N. costalis*, defensive recruitment is mediated by the trail pheromone pro-

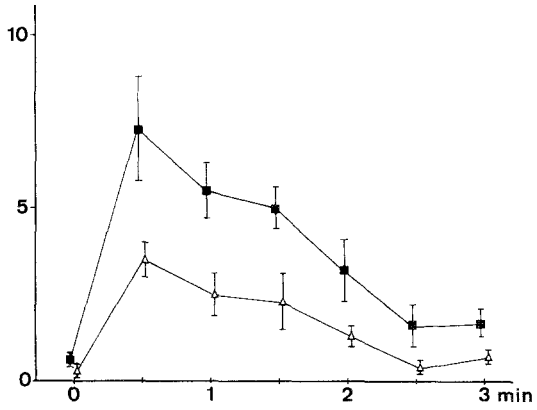


FIG. 5. Attractiveness of (+)-α-pinene in 1% hexane solution (■) to LW2+ compared with hexane blank (△). Number of LW2+ around source over time. Mann-Whitney U test: $P < 0.05$ (0.5 min), $P < 0.01$ (1–1.5 min), NS (2–3 min).

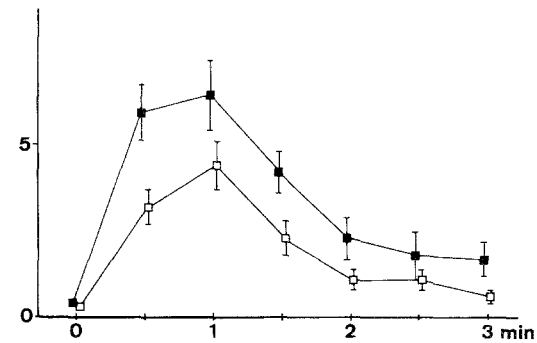


FIG. 6. Compared attractiveness of 1% hexane solutions of (+)-α-pinene (■) and (−)-α-pinene (□) to LW2+. Number of LW2+ around source over time. Mann-Whitney U test: $P < 0.01$ (0.5 min), $P < 0.05$ (1.5 min), NS (1 min, 2–3 min).

duced by the sternal gland of the soldiers, whereas both "frontal and sternal gland pheromones have a pronounced effect on arresting motion" (Traniello, 1981). In *N. exitiosus*, soldiers converge toward an item sprayed with soldier secretion; the workers, however, are much less attracted than soldiers (Eisner et al., 1976). In *N. corniger*, only soldiers are attracted by the secretion, while workers remain in or retreat to the nest (Stuart, 1981).

In contrast, in *N. princeps*, both large workers and soldiers are attracted by the soldier secretion, but the two castes react in a different way, which can be correlated to their complementary roles in defense. Excited soldiers patrol

the vicinity of the source over a broader area than the workers, which are more precisely attracted toward the source of monoterpene. This reaction of the soldiers would increase the probability of encounters with mobile enemies. Interestingly, the release of their secretion is not triggered by an immobile item, even impregnated by their secretion. This contrasts with the observations reported by Ernst (1959) and Vrkoc et al. (1978), but agrees with those of Eisner et al. (1976) and Traniello (1981). The role of the workers would be to eliminate the enemies immobilized by the soldiers. Indeed, the workers often isolate the source of disturbance by covering it with building material, and/or by constructing partitions. This, of course, does not imply that the workers will not participate in defense by biting an enemy with their reach. Several authors have shown that workers can be good fighters, especially efficient in termite–termite contests (Andrews, 1911; Eisner et al., 1976; Thorne, 1982; Traniello and Beshers, 1985). The much lower reaction of large workers in mixed groups including soldiers is intriguing. It suggests that workers can perceive, at very close range and by another cue that the secretion, the presence of soldiers and will react mainly after soldiers have been mobilized. A comparable delayed reaction of workers was reported by Traniello (1981) during food recruitment in *N. costalis*. In this species, the differential responses of soldiers and workers depend on their reaction threshold to the trail pheromone: workers need a higher concentration of trail pheromone than soldiers to leave the nest (Traniello and Busher, 1985).

In this paper, we have investigated the short-range reaction to defensive secretion, but not a possible long-range recruitment to the site of disturbance. Defensive recruitment by trail pheromone in *N. princeps* remains a possibility, which was not tested by our experimental setting.

The nest structure of arboreal *Nasutitermes*, characterized by wide openings between adjacent chambers, is especially suitable for an airborne short-range communication system, allowing rapid diffusion of the recruitment pheromone as well as troop movements (Deligne and Pasteels, 1982). An exactly opposite situation occurs in the mandibulate Nasutitermitinae *Armitermes chagresi*, whose soldiers block the narrow passages between nest chambers with their head: their defensive secretion consists of low volatile macrocyclic lactones devoid of pheromonal properties (Traniello et al., 1984).

The weak reaction of young, either large or small, workers could be expected. In species where several worker subcastes are present, some division of labor is common. Such a polyethism can involve trail-laying and trail-following (Pasteels, 1965; Traniello, 1981), nest repair, foraging (McMahan 1970, 1977; Watson and McMahan, 1978), and food gathering and processing (Badertscher et al., 1983). As a rule, the older, most pigmented workers perform most of the external chores, while the youngest ones usually work inside the

nest. Accordingly, it is not surprising that the older large workers are by far the most responsive worker subcaste to the soldier secretion in *N. princeps*.

The attractive action of the secretion of *N. princeps* soldiers can be reproduced by a 1% solution of (+)-α-pinene in hexane. During our tests, the workers' response was, however, less massive and subsided sooner than with natural secretion, although the amount of (+)-α-pinene presented in solution was comparable to the average content of three soldier heads. This can be due either to a disturbing effect of the solvent or to the absence of the other components present in the natural secretion: diterpenes could be important in reducing the evaporation of the monoterpenes, and so maintain a suitable gradient for a longer period of time, whereas minor monoterpenes (limonene, α-thujene, sabinene, myrcene, α-phellandrene, α-terpinene, and terpinolene) might enhance the effect of (+)-α-pinene synergistically.

In the present work, the pheromonal properties of (+)-α-pinene are clearly demonstrated in *N. princeps*: despite identical physical properties, it is more active than its enantiomer, which is almost absent from the soldier cephalic secretion. This is the first time that a differential reaction to both enantiomers of the same molecule has been demonstrated for an alarm/recruitment pheromone in termites, although this was recently reported for alarm pheromones in ants (Cammaerts et al., 1985; Cammaerts and Mori, 1987), and for potential trail pheromone of some *Nasutitermes*: in *N. costalis*, Hall and Traniello (1985) tested the recruitment effects of racemic and chiral cembrene-A, a substance identified as the trail pheromone of *N. exitiosus* and which could possibly play the same role in some other termite species. Although the recorded responses were very low, the chiral compound was more active that the racemic one.

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CONFORMATIONAL ANALYSIS OF SERRICORNIN: APPLICATION OF MOLECULAR MECHANICS CALCULATIONS TO STEREOCHEMICAL ASSIGNMENT OF SERRICORNIN, SEX PHEROMONE OF CIGARETTE BEETLE (*Lasioderma serricorne* F.)

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Abstract—Conformational analysis using molecular mechanics (MM) was performed for a determination of the stereochemistry of serricornin, the sex pheromone of the cigarette beetle (*Lasioderma serricorne* F.). An exhaustive conformational analysis using MM2 calculations with algorithms for covering torsional energy surfaces of flexible molecules furnishes coordinates and steric energies of all local energy minimum conformers of serricornin, both acyclic and the corresponding cyclic forms. These coordinates gave angles required for the calculation of vicinal H/H coupling constants ($^3J_{HH}$s) of each energy minima by Altona's modified Karplus equation. The Boltzmann distributions of all local energy minima were calculated from their steric energies to furnish populations of each energy minimum conformer. Population-weighted averaged $^3J_{HH}$s of four enantiomeric pairs, (*S**, *S**, *S**)-, (*S**, *S**, *R**)-, (*S**, *R**, *S**)-, and (*R**, *S**, *S**)-serricornins were calculated from

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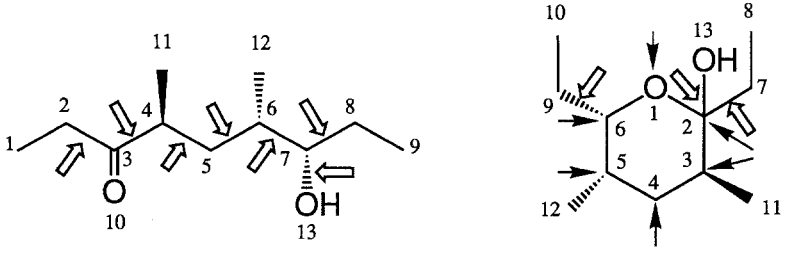
the data above. The observed $^3J_{HH}$s of the naturally occurring serricornin, both acyclic and cyclic forms, are fitted best to calcd. $^3J_{HH}$s of (4*S**, 6*S**, 7*S**)-acyclic and (3*S**, 5*S**, 6*S**)-cyclic serricornin, respectively, among those of four enantiomeric pairs of serricornin.

Key Words—Conformational analysis, molecular mechanics, determination of absolute configuration, sex pheromone, *Lasioderma serricorne* F., Coleoptera, Anobiidae, cigarette beetle, serricornin.

INTRODUCTION

Serricornin, a female-produced sex pheromone of the cigarette beetle, was isolated and structurally elucidated to be an equilibrating mixture of 4,6-dimethyl-7-hydroxynonan-3-one and the corresponding cyclic hemiketal (Figure 1) (Chuman et al. 1985). Many efforts on synthetic studies of the diastereomeric mixture (Chuman et al., 1979), (4*RS*, 6*S*, 7*R*)- (Chuman et al., 1981), (4*RS*, 6*R*, 7*R*)- (Mori et al., 1981), (4*RS*, 6*S*, 7*S*)- (Hoffmann et al., 1982), (4*S*, 6*R*, 7*R*)- (Mori et al., 1982a), and (4*S*, 6*S*, 7*S*)-acyclic serricornins (Mori et al., 1982b; Mori and Watanabe, 1985) were required to established the absolute configuration of naturally occurring serricornin to be (4*S*, 6*S*, 7*S*) as the acyclic form and (3*S*, 5*S*, 6*S*) as the cyclic form (Chuman et al., 1985). The determination of the absolute configuration is important to develop an effective lure for the pest, because only (*S*, *S*, *S*)-serricornin elicits pheromone activity among eight possible stereoisomers (Chuman et al., 1985).

This study focused on a determination of the absolute configuration of serricornin using molecular mechanics (MM) calculations. It is revealed that MM conformational analysis is useful for the convenient determinations of unknown stereochemistry of natural products.



(4*S*,6*S*,7*S*)-ACYCLIC SERRICORNIN **(3*S*,5*S*,6*S*)-CYCLIC SERRICORNIN**

FIG. 1. Cyclic and acyclic structures of serricornin. White arrows and black arrows point to bonds that were rotated by TREE and corners that were flapped by CONFLEX, respectively.

METHODS AND MATERIALS

Molecular Mechanics Calculation for Conformational Analysis. The MM2 (Burkert and Allinger, 1982; Version '85: Molecular Design Limited, USA) molecular mechanics program is used in structural optimization with QUANTA (Polygen Co., Waltham, Massachusetts) as a computer graphics program running on IRIS-4D/80GT (Silicon Graphics Computer Systems Inc., Mountain View, California). Introduction of the initial two-dimensional structure was performed by the CHEMNOTE option in the QUANTA program.

Exhaustive conformational analysis by generation of all possible conformation of acyclic and cyclic serricornins was performed by the TREE (Masamune et al., 1986) and CONFLEX (Gotō and Ōsawa, 1989) programs, respectively. Acyclic and cyclic candidates generated by these option programs were structurally optimized by MM2. The Boltzmann population of each energy minimum was calculated from their steric energies. Population-weighted vicinal H/H coupling constants ($^3J_{HH}$s) were calculated by the $^3J_{HH}$ program (Jaime et al., 1987), which includes Altona's empirical modification of Karplus equation (Haasnoot et al., 1980).

Procedure of Conformational Analysis of Serricornin. All possible candidates of acyclic serricornin for MM optimization were generated by the TREE program from the combination of staggered arrangements, anti, gauche(+) and gauche(−) on each backbone C2−C3, C3−C4, C4−C5, C5−C6, C6−C7, C7−C8, and C7−O13 bonds[5] (Figure 1). All possible candidates of the cyclic form for MM optimization also were generated by the CONFLEX program from the combinations of pyran ring conformations (chair, boat, and twist), with staggered arrangements (C2−C7, C6−C9, C2−O13), and with orientations of hydroxyl group (axial and equatorial) (Figure 1).

All conformations generated were subjected to MM structural optimization to give coordinates and steric energies of each energy minimum. The Boltzmann distributions of each energy minimum were calculated from the steric energy. These conformational analyses were performed for four enantiomeric pairs of both acyclic and cyclic serricornin.

Comparison of vicinal H/H coupling constants ($^3J_{HH}$s). Vicinal H/H coupling constants ($^3J_{HH}$s) were calculated by Altona's empirical modification of Karplus equation (Haasnoot et al., 1980). Coordinates of each energy minimum bring angle information required for the $^3J_{HH}$ calculation in the above equation. Population-weighted averaged $^3J_{HH}$ values of four enantiomeric pairs of serricornin were obtained from the calculation using $^3J_{HH}$s for each conformer and their Boltzmann distributions.

[5] In this study, anti, gauche(+) and gauche(−) conformations were generated on two C(sp^3)−C(sp^2) bonds (C2−C3 and C3−C4 bonds) as well as other C(sp^3)−C(sp^3) bonds by TREE program.

All NMR signals of naturally occurring serricornin were completely assigned to protons belonging to both the acyclic and cyclic structures independently by COSY-NMR, although the NMR spectrum was complicated because of the acyclic–cyclic equilibrium (Mori et al., 1984). Thus, the $^3J_{HH}$s of natural occurring acyclic and cyclic serricornins can be compared to the corresponding MM calculated $^3J_{HH}$s of (*S**, *S**, *S**)[6]-, (*S**, *S**, *R**)-, (*S**, *R**, *S**)-, and (*R**, *S**, *S**)-acyclic/cyclic serricornins.

RESULTS AND DISCUSSION

Conformation Analysis of Acyclic Form. Two thousand one hundred eighty-seven ($=3^7$) candidates of acyclic serricornin for MM optimization were generated by the combination of the staggered arrangements of the seven bonds (Figure 1). MM optimization of these candidates of (4*S**, 6*S**, 7*S**)-, (4*S**, 6*S**, 7*R**)-, (4*S**, 6*R**, 7*S**)-, and (4*R**, 6*S**, 7*S**)-acyclic serricornin furnished 1165, 1227, 1409, and 1164 local energy minima, respectively (Table 1). In the case of (4*S**, 6*S**, 7*S**)-serricornin, 19 conformations among these 1165 conformations have over 1% population by Boltzmann distribution calculation. The major conformer has an anti(C2—C3), anti(C3—C4), (+)-gauche(C4—C5), (+)-gauche(C5—C6), (+)-gauche(C6—C7), anti(C7—C8), and (−)-gauche(C6—O13) conformation (Figure 2). The population of the major conformer was ca. 10%, and many local energy minima with small population exist within Δ1.0 kcal/mol (Table 1). (4*S**, 6*S**, 7*S**)-Acyclic serricornin has no big major conformation, according to MM calculations. Fifteen majors in these 19 conformers have gauche conformations on the middle part of the molecule, C4—C5 and/or C5—C6 bonds, while conformers having anti conformations in these bonds, like the all-anti conformer (No. 17 conformer), are very few (Ōsawa et al. 1989). The predominant shape of the molecule is a folded type, not an extended type (Figure 2). The tendency is the same in the stable conformations of other acyclic serricornin isomers (Table 1). The MM2 program used in this study has no function for energy estimation of hydrogen bonding for the carbonyl and hydroxyl groups in the acyclic serricornin. By addition of this function on the MM calculation, the folded-type molecule may be estimated more positively as the stable conformation.

Conformational Analysis of Cyclic form. The CONFLEX program for generation of all possible conformers of cyclic serricornin produced candidates for MM optimization. MM optimization of all those candidates of (3*S**, 5*S**, 6*S**)-, (3*S**, 5*S**, 6*S**)-, (3*S**, 5*R**, 6*S**)-, and (3*R**, 5*S**, 6*S**)-cyclic serricornin furnished 303, 213, 277, and 260 local energy minima, respectively (Table 2). In contrast to the case of acyclic serricornin, the major conformers

[6] A stereochemical designation with asterisks such as (*S**, *S**, *S**) means the enantiomeric pair (*S*, *S*, *S*) and (*R*, *R*, *R*).

TABLE 1. CONFORMATIONAL ANALYSIS OF ACYCLIC SERRICORNIN

Conf. No.	Torsions 2-3	3-4	4-5	5-6	6-7	7-8	7-13	ΔSteric E (kcal/mol)	Population (%)
(4*S*,6*S*,7*S*)/(4*R*,6*R*,7*R*)-acyclic serricornin[a]									
1	A	A	G(+)	G(+)	G(+)	A	G(−)	0.00	10.3
2	A	G(−)	G(−)	G(+)	A	A	A	0.23	7.0
3	A	A	G(+)	G(+)	G(+)	A	A	0.44	4.9
4	A	G(−)	G(−)	G(+)	A	G(+)	A	0.50	4.4
5	A	G(+)	G(+)	G(+)	A	A	A	0.52	4.3
6	A	G(+)	G(+)	G(+)	A	G(+)	A	0.62	3.6
7	A	A	G(+)	G(−)	A	A	A	0.78	2.7
8	A	G(−)	A	A	A	A	A	0.81	2.6
9	A	A	G(+)	G(+)	A	A	A	0.83	2.5
10	A	A	G(+)	G(+)	A	G(+)	A	0.93	2.1
11	A	A	G(+)	G(+)	A	A	G(+)	1.08	1.7
12	G(+)	A	G(+)	G(+)	G(+)	A	G(−)	1.12	1.6
13	A	G(−)	A	A	A	G(+)	A	1.16	1.5
14	A	G(+)	G(+)	G(+)	A	A	G(−)	1.19	1.4
15	A	A	G(−)	G(+)	A	A	G(−)	1.19	1.4
16	A	A	A	A	A	A	G(−)	1.20	1.4
17[b]	A	A	A	A	A	A	A	1.28	1.2
18	A	A	G(+)	G(+)	A	G(+)	G(+)	1.32	1.1
19	A	A	G(+)	G(+)	A	A	G(−)	1.36	1.0
(4*S*,6*S*,7*R*)/(4*R*,6*R*,7*S*)-acyclic serricornin[c]									
1	G(−)	A	G(+)	A	A	A	A	0.00	5.6
2	G(−)	A	G(+)	A	A	G(+)	A	0.21	3.9
3	G(+)	A	A	G(+)	G(+)	A	G(+)	0.33	3.2
4	A	A	A	G(+)	G(+)	A	G(−)	0.35	3.1
5	G(−)	A	G(−)	A	A	A	G(+)	0.36	3.1
6	G(+)	A	G(+)	G(+)	G(+)	A	A	0.39	2.9
7	G(+)	A	G(+)	G(+)	G(+)	A	G(+)	0.44	2.7
8	G(−)	A	G(−)	A	A	G(−)	G(+)	0.60	2.0
9	G(−)	A	A	A	A	A	A	0.62	2.0
[b]	A	A	A	A	A	A	A	1.16	0.8
(4*S*,6*R*,7*S*)/(4*R*,6*S*,7*R*)-acyclic serricornin[d]									
1	G(+)	A	A	A	G(+)	A	A	0.00	4.9
2	G(+)	A	A	G(+)	G(+)	A	A	0.14	3.9
3	G(+)	G(+)	A	A	G(+)	A	A	0.14	3.9
4	G(+)	A	A	G(+)	G(+)	A	G(−)	0.21	3.5
5	A	A	A	A	G(+)	G(−)	A	0.26	3.2
6	G(+)	A	A	A	G(+)	A	G(−)	0.29	3.0
7	G(+)	G(+)	A	G(+)	G(+)	A	A	0.30	3.0
8	G(+)	G(+)	A	G(+)	G(+)	A	G(−)	0.39	2.5
9	G(−)	A	A	G(−)	A	G(−)	A	0.48	2.2
10	G(+)	G(+)	A	A	G(+)	A	G(−)	0.50	2.1
11	G(−)	A	A	G(−)	A	G(−)	G(−)	0.50	2.1
12	A	A	A	G(+)	G(+)	A	A	0.51	2.1
[b]	A	A	A	A	A	A	A	3.48	0.01

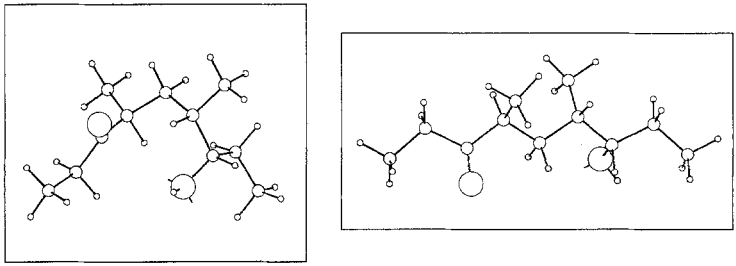
TABLE 1. Continued

Conf. No.	Torsions 2-3	3-4	4-5	5-6	6-7	7-8	7-13	ΔSteric E (kcal/mol)	Population (%)
(4*R*,6*S*,7*S*)/(4*S*,6*R*,7*R*)-acyclic serricornin[c]									
1	A	G(+)	A	G(+)	A	A	A	0.00	5.2
2	A	A	G(−)	G(+)	A	A	G(−)	0.05	4.8
3	A	G(+)	A	G(+)	A	G(+)	A	0.13	4.2
4	A	A	G(−)	A	A	A	A	0.28	3.2
5	A	A	G(−)	A	G(+)	A	G(−)	0.30	3.1
6	A	G(−)	G(−)	A	G(+)	A	A	0.38	2.8
7	A	A	G(−)	A	A	A	G(−)	0.55	2.0
8	A	A	G(−)	G(+)	A	G(+)	G(−)	0.56	2.0
[b]	A	A	A	A	A	A	A	2.72	0.05

[a] The total number of local energy minima obtained: 1165. Only conformers with over 1% population are represented.
[b] All-anti conformer.
[c] The total number of local energy minima obtained: 1227. Only conformers with over 2% population are represented.
[d] The total number of local energy minima obtained: 1409. Only conformers with over 2% population are represented.
[e] The total number of local energy minima obtained: 1164. Only conformers with over 2% population are represented.

have large populations, and the total population is composed of several energy minima, although over 200 energy minima were obtained in the conformation analysis of each enantiomeric pair of cyclic serricornin (Table 2).

It is worth mentioning that all the major conformers of (3*S*, 5*S*, 6*S*)-



Folded Conformation
The major conformation: 10.3%

Extended Conformation
The 17th energy minimum: 1.2%

FIG. 2. Folded and extended structures of (4*S*, 6*S*, 7*S*)-acyclic serricornin. Large, medium, and small circles indicate oxygen, carbon, and hydrogen atoms, respectively. Lone pairs of electrons are represented by lines from oxygen atoms.

TABLE 2. CONFORMATIONAL ANALYSIS OF CYCLIC SERRICORNIN

Conf. No.	Ring Conf.	C2 Config.	Orient. of OH	Torsions 2-7	Torsions 2-13	Torsions 6-9	ΔSteric E (kcal/mol)	Population (%)
(3*S*,5*S*,6*S*)/(3*R*,5*R*,6*R*)-cyclic serricornin[a]								
1	$^4C_1$ chair	*R*/*S*[b]	Axial	G(+)	G(−)	G(−)	0.00	47.5
2	$^4C_1$ chair	*R*/*S*	Axial	G(−)	G(−)	G(−)	0.36	26.0
3	$^4C_1$ chair	*R*/*S*	Axial	G(+)	G(−)	A	0.77	12.9
4	$^4C_1$ chair	*R*/*S*	Axial	G(−)	G(−)	A	1.01	8.6
*	$^4C_1$ chair	*R*/*S*	Equat.	G(−)	G(−)	G(−)	3.27	0.3
(3*R*,5*R*,6*S*)/(3*S*,5*S*,6*R*)-cyclic serricornin[c]								
1	$^4C_1$ chair	*R*/*S*[d]	Axial	G(+)	G(+)	G(−)	0.00	44.3
2	$^4C_1$ chair	*R*/*S*	Axial	G(+)	G(+)	A	0.38	23.3
3	$^4C_1$ chair	*R*/*S*	Axial	G(−)	G(+)	G(−)	0.72	13.1
4	$^4C_1$ chair	*R*/*S*	Axial	G(−)	G(+)	A	1.01	8.1
5	$^4C_1$ chair	*R*/*S*	Axial	G(+)	A	G(−)	1.84	2.0
(3*S*,5*R*,6*S*)/(3*R*,5*S*,6*R*)-cyclic serricornin[e]								
1	$^4C_1$ chair	*R*/*S*[f]	Axial	G(+)	G(−)	G(−)	0.00	48.6
2	$^4C_1$ chair	*R*/*S*	Axial	G(−)	G(−)	G(−)	0.41	24.3
3	$^4C_1$ chair	*R*/*S*	Axial	G(+)	G(−)	G(+)	0.76	13.4
4	$^4C_1$ chair	*R*/*S*	Axial	G(−)	G(−)	G(+)	1.01	8.9
(3*R*, 5*S*, 6*S*)/(3*S*, 5*R*, 6*R*)-cyclic serricornin[g]								
1	$^1C_4$ chair	*S*/*R*[h]	Axial	G(−)	G(+)	G(−)	0.00	29.4
2	$^4C_1$ chair	*R*/*S*	Axial	G(+)	G(−)	G(−)	0.46	13.6
3	$^1C_4$ chair	*S*/*R*	Axial	G(+)	G(+)	G(−)	0.49	12.9
4	Twist	*R*/*S*	Axial	G(+)	G(−)	G(−)	0.49	12.8
5	$^4C_1$ chair	*R*/*S*	Axial	A	G(−)	G(−)	1.02	5.3
6	$^4C_1$ chair	*R*/*S*	Axial	G(+)	G(−)	A	1.23	3.7
7	Twist	*R*/*S*	Axial	G(+)	G(−)	A	1.33	3.1
8	$^1C_4$ chair	*S*/*R*	Axial	G(−)	G(−)	G(−)	1.51	2.3

[a] The total number of local energy minima obtained: 303. Only conformers with over 2% population are represented.
[b] *R*-configuration in (*S*,*S*,*S*) and *S*-configuration in (*R*,*R*,*R*).
[c] The total number of local energy minima obtained: 213. Only conformers with over 2% population are represented.
[d] *R*-configuration in (*R*,*R*,*S*) and *S*-configuration in (*S*,*S*,*R*).
[e] The total number of local energy minima obtained: 277. Only conformers with over 2% population are represented.
[f] *R*-configuration in (*S*,*R*,*S*) and *S*-configuration in (*R*,*S*,*R*).
[g] The number of local energy minima obtained: 260. Only conformers with over 2% population are represented.
[h] *S*-configuration in (*R*,*S*,*S*) and *R*-configuration in (*S*,*R*,*R*).

sericornin have a common $^4C_1$ chair ring conformation with *R*-configuration at C2, which has an axial hydroxyl group (Figure 3).

The major conformation at C2—C7, C2—O13, and C6—C9 torsions are (+)-, (−)-, and (−)-gauche, respectively. The population of the major conformation having an equatorial hydroxyl at C2 is only 0.3% (Table 2). These characteristics of the stable conformation are common to those of (3*R*, 5*R*, 6*S*)- and (3*S*, 5*R*, 6*S*)-cyclic sericornins, which have the $^4C_1$ chair pyran ring with the *R*-configuration at C2 having the axial hydroxyl group (Table 2, Figure 4).

The X-ray crystallographic analysis (Mori et al, 1984) of the (3*S*, 5*R*, 6*S*)-cyclic sericornin resulted in the same $^4C_1$ chair conformation with *R*-con-

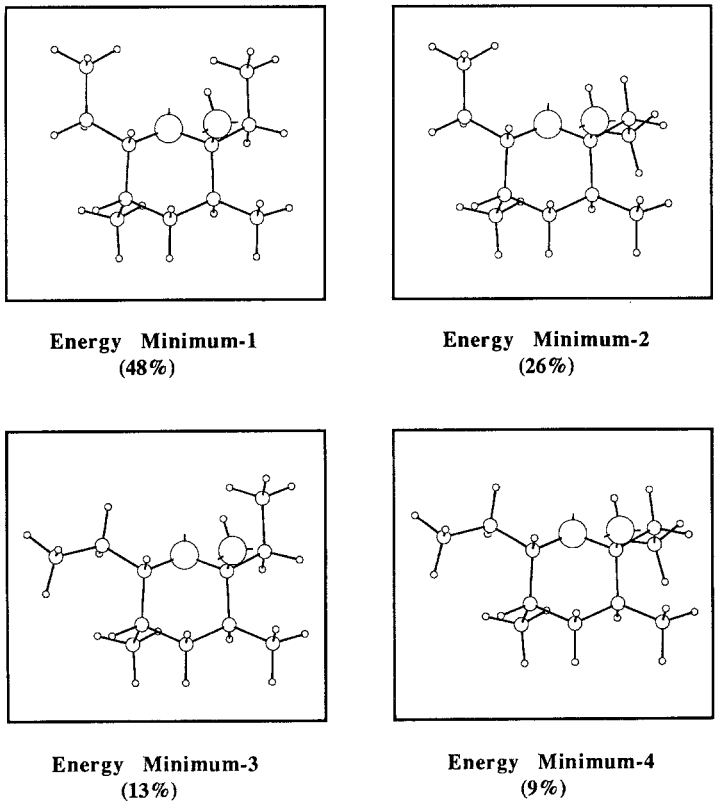


FIG. 3. Major conformations of (3*S*, 5*S*, 6*S*)-cyclic sericornin. Large, medium, and small circles indicate oxygen, carbon, and hydrogen atoms, respectively. Lone pairs of electrons are represented by lines from oxygen atoms.

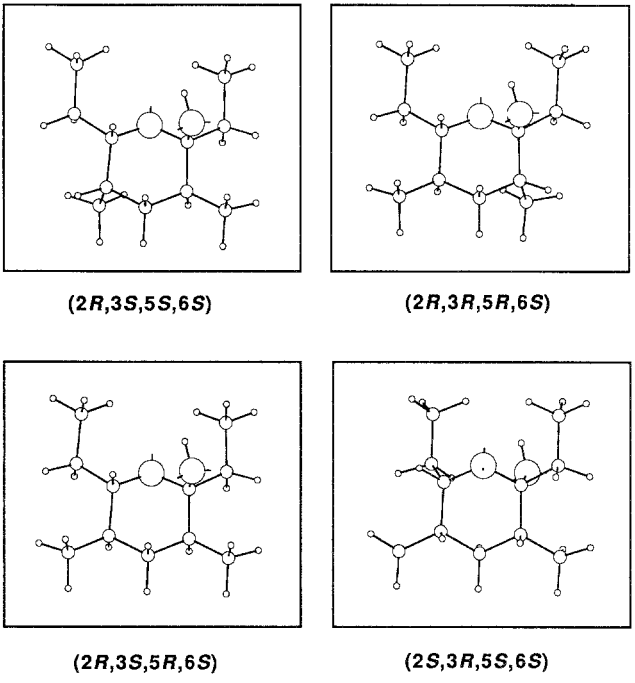


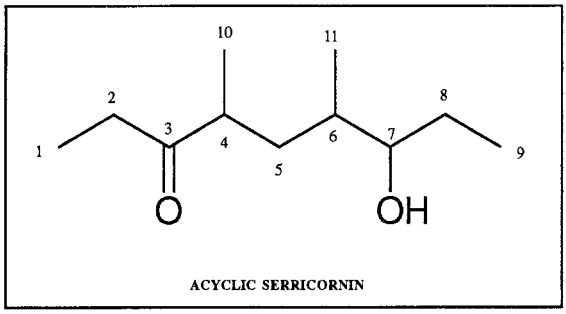
FIG. 4. The major conformations of stereoisomers of cyclic serricornin. Large, medium, and small circles indicates oxygen, carbon, and hydrogen atoms, respectively. Lone pairs of electrons are represented by lines from oxygen atoms.

figuration at C2 as that assigned by MM conformational analysis, although there are differences in the torsion of the side chains at C2—C7 and C6—C9.

The major conformations of (3*R*, 5*R*, 6*R*)-, (3*S*, 5*R*, 6*R*)-, and (3*R*, 5*S*, 6*R*)-cyclic serricornins, enantiomers of the above 6*S*-isomers, are $^1C_4$ chairs with *S*-configuration at C2 having the axial hydroxyl (Table 2).

Conformational analysis of (3*R*, 5*S*, 6*S*)-cyclic serricornin gave the major conformation having $^1C_4$ chair conformation with *S*-configuration at C2 having an axial hydroxyl (Figure 4). Two twist ring conformations also existed in the major group (Table 2). The major conformation of (3*S*, 5*R*, 6*R*)-cyclic serricornin, therefore, has $^4C_1$ chair conformation with *R*-configuration at C2 having an axial hydroxyl. The molecule tries to avoid the 1,3-diaxial relationship of two methyls in the $^4C_1$ or $^1C_4$ chair conformation, resulting in the 1,3-diequatorial relationship in the $^1C_4$ or $^4C_1$ chair (Table 2).

Stereochemical Assignment of Naturally Occurring Serricornin by Comparison between Calcd. and Observed $^3J_{HH}$s Values. The comparative study on



ACYCLIC SERRICORNIN

CALCULATED $^3J_{HH}$ VALUES (H_Z) OF CHAIN SERRICORNIN

STEREO (4,6,7)	J_{4-5}	$J_{4-5'}$	J_{5-6}	$J_{5'-6}$	J_{6-7}	J_{7-8}	$J_{7-8'}$	RMS
SSS/RRR	5.47	8.27	8.66	6.16	2.75	8.91	4.01	0.83
SSR/RRS	7.67	6.75	6.03	8.49	4.39	3.66	8.90	2.82
SRS/RSR	9.50	4.37	5.08	9.29	4.15	3.80	8.85	3.19
RSS/SRR	8.02	6.11	7.28	7.32	2.98	8.69	4.15	1.27

OBSERVED $^3J_{HH}$ VALUES (H_Z) OF NATURAL SERRICORNIN

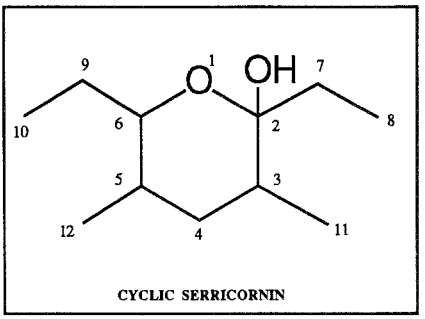
NATURAL OCCURRING	6.20	7.60	8.10	5.40	3.80	8.50	5.00

FIG. 5. MM calculated $^3J_{HH}$s of four enantiomeric pairs of acyclic serricornin and observed $^3J_{HH}$s of naturally occurring acyclic serricornin.

vicinal H/H coupling constants ($^3J_{HH}$s) between those observed in the NMR and those calculated by Altona's modified Karplus equation was conducted for an assignment of the unknown stereochemistry of naturally occurring serricornin (Figures 5 and 6).

As has been shown previously (Masamune et al., 1986), this technique produces rather large errors, but simple root-mean-square tests (the right columns of Figures 5 and 6) unambiguously show that the observed $^3J_{HH}$s pattern of both acyclic and cyclic natural serricornin fitted best with those calculated for the (4*S**, 6*S**, 7*S**)-acyclic serricornin and (3*S**, 5*S**, 6*S**)-cyclic serricornin, respectively.

If this information had been known before starting the synthetic work, it may have been unnecessary to synthesize all the stereoisomers but (*S*, *S*, *S*)- or (*R*, *R*, *R*)-serricornins for determination of the absolute configuration. This



CYCLIC SERRICORNIN

CALCULATED $^3J_{HH}$ VALUES (H_Z) OF CYCLIC SERRICORNIN

STEREO (3,5,6)	J_{3-4}	$J_{3-4'}$	J_{4-5}	$J_{4'-5}$	J_{5-6}	J_{6-9}	$J_{6-9'}$	RMS
SSS/RRR	3.96	12.22	1.98	4.54	2.24	9.36	3.66	1.48
SSR/RRS	2.07	4.69	3.71	12.11	10.19	9.28	2.65	5.26
SRS/RSR	3.64	12.30	3.38	12.34	10.21	9.32	2.69	5.59
RSS/SRR	9.32	4.01	8.37	5.14	4.01	3.12	10.24	5.05

OBSERVED $^3J_{HH}$ VALUES (H_Z) OF NATURAL SERRICORNIN

NATURAL OCCURRING	2.50	12.50	4.10	4.30	2.60	7.80	6.10

FIG. 6. MM calculated $^3J_{HH}$s of four enantiomeric pairs of cyclic serricornin and observed $^3J_{HH}$s of naturally occurring cyclic serricornin.

work indicates that MM conformational analysis can bring effective information for the determination of the unknown stereochemistry in natural products.

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PREFERENCES OF MATED *Heliothis virescens* AND *H. subflexa* FEMALES FOR HOST AND NONHOST VOLATILES IN A FLIGHT TUNNEL[1]

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Abstract—In flight-tunnel bioassays, mated female *Heliothis subflexa* (Gn.) moths demonstrated in dual-choice tests a significant preference for volatiles from an extract of their only known host, *Physalis* spp. (groundcherry). However, *H. virescens* (F.), a polyphagous species, responded positively by anemotaxis to extracts from susceptible tobacco, cotton, *Desmodium tortuosum* (host plants), and groundcherry, a nonhost. *H. virescens* females did not fly to volatiles emanating from an extract of a resistant tobacco cultivar.

Key Words—Cotton, tobacco, groundcherry, *Physalis*, *Desmodium*, *Heliothis subflexa*, *Heliothis virescens*, Lepidoptera, Noctuidae, plant–insect interaction, host-plant resistance, attractant.

INTRODUCTION

The recent development of environmentally controlled systems for studying host-finding and ovipositional behaviors of phytophagous insects has made possible the identification and evaluation of these behaviors and the isolation of the chemical components of plants that attract insect pests for oviposition. The ovipositional responses of three *Heliothis* species to allelochemicals from cultivated and wild host plants in a "choice" bioassay system recently was reported

[1] This article reports the results of research only. Mention of a proprietary product does not constitute an endorsement or the recommendation of its use by USDA.

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by Mitchell et al. (1990). In the olfactometer developed by Mitchell and Heath (1987), they evaluated cuticular washes from tobacco, cotton, and *Desmodium tortuosum*, host plants of *H. virescens* (F.) and *H. zea* (Boddie), and from groundcherry (*Physalis angulata*), the host plant of *H. subflexa* (Gn). They found that extracts from tobacco leaves elicited the highest level of ovipositional stimulation in *H. virescens*, and the extract from groundcherry leaves showed the highest level of stimulation for *H. subflexa*; none of the extracts stimulated oviposition by *H. zea*. Earlier, Jackson et al. (1984) reported that whole-leaf cuticular washes from green leaves of a flue-cured tobacco stimulated oviposition by *H. virescens* when sprayed onto leaves of a resistant tobacco strain. Also, Rembold and Tober (1985) showed differential attraction by *H. armigera* (Hb.) females to volatiles from resistant and susceptible varieties of pigeonpea.

Tingle et al. (1989) demonstrated in flight-tunnel bioassays that mated female *H. subflexa* showed positive anemotaxis (i.e., oriented flight toward the odor source) in the presence of an extract from groundcherry. Furthermore, Mitchell et al. (unpublished data) have observed that mated female *H. virescens* respond by positive anemotaxis in the flight tunnel to extracts from cotton, tobacco, and *Desmodium*. The purpose of the present study was to determine if mated *H. virescens* and *H. subflexa* females would demonstrate, by anemotaxis, a preference for volatiles in the flight tunnel when exposed simultaneously to extracts from cultivated and wild host and nonhost plants.

METHODS AND MATERIALS

A Plexiglas flight tunnel (60 × 60 × 195 cm) was used in dual-choice tests to observe the preference and flight responses of *H. virescens* and *H. subflexa* to volatiles from crude plant extracts of host and nonhost plants for each species. The extracts were obtained by washing ca. 400 g fresh plant material for 30 sec in 1 liter of solvent, which was filtered and stored in 1-liter glass containers at 0°C until needed (Mitchell and Heath, 1987). Methanol was used as the solvent for washing whole leaves of groundcherry (*P. angulata*), and methylene chloride was used for washing all other plant material (Mitchell et al., 1990). Extracts were prepared from cotton (variety McNair 220) leaves and squares (flower buds) and from leaves of *D. tortuosum* and tobacco (susceptible: NC 2326, and resistant: TI 1112). Before testing, the extracts were concentrated in a rotary evaporator to 1 gram equivalent (gE) of plant material per 200 μl solvent. All plants were in the flowering stage except tobacco, which was in the prebloom stage. The flowering stage of host plants has been shown as the most preferred phenological state by *H. zea* (Johnson et al., 1975) and *H. armigera* (Roome, 1975).

All test insects were reared in our laboratory colonies on modified pinto

bean diet using the methods described by Guy et al. (1985) and Mitchell et al. (1988). The *H. subflexa* colony was started in 1983 (Mitchell et al., 1988) and the *H. virescens* colony was started in 1987 (Mitchell et al., unpublished data) from field-collected insects; new genetic material from the field has been added each year since then. For testing, insects were sexed in the pupal stage and held separately in 3.8-liter paper cans with screened tops until emergence. Upon eclosion, the adults were confined for mating (21 females and 14 males/cage) in 5.5-liter plastic cages with screened tops and held under a reversed 14-hr light–10-hr dark cycle in a holding room that was maintained at 25–26°C and 60–70% relative humidity.

On the day of testing, the moths were sexed by gently squeezing the abdomen to extrude the genitalia. The females were then confined in a 25 × 25 × 25-cm Plexiglas holding cage and placed in the flight-tunnel room (3 × 2.6 × 2.1 m) ca. 1 hr before scotophase. An intake vent in the wall allowed a continuous flow of fresh unfiltered air from the outside into the room, which was maintained at the same environmental conditions as the holding room. An electric timer was used to control overhead fluorescent lights (two banks of two 40-W bulbs). Three 25-W red light bulbs that were equally spaced above the tunnel remained on continuously and provided a light level of 2.4 lux inside the tunnel. Air was pulled through the tunnel at ca. 0.4 m/sec when measured at the center of the tunnel and exhausted via a 30-cm-diam. flexible pipe with a fan. Except for slight modifications, the flight tunnel was the same as the tunnel used by Tingle et al. (1989). For these experiments, a V-shaped Plexiglas baffle was placed on each side of the flight tunnel so that the plume trails from two dispensers would combine into one plume at the desired location. The baffles were located 124 cm from the upwind end of the tunnel and 71 cm from the downwind end with a horizontal distance of 28 cm between them.

A test was begun by introducing separately and simultaneously 1-gE of two selected plant extracts into the tunnel on evaporator substrates (white muslin) that covered the ends of glass cylinder dispensers with 3.5-cm openings. After a 5-min evaporation time, the two dispensers were placed 30-cm apart at the upwind end of the tunnel and were rotated after each replication. Air was blown through each cylinder with an aquarium pump at the rate of 1 liter/min to provide a continuous flow (plume) of the extract odor through the tunnel. The location and form of the plumes were verified previously by introducing smoke into the dispenser system and observing the smoke trails. The plume from each dispenser remained separate until it combined with the plume from the other dispenser midway through the tunnel ca. 25 cm before passing between the baffles.

Each moth was removed from the holding cage and placed into a cylindrical 4 × 6.5-cm plastic cage with screened ends. After placement of the release cage into the downwind end of the flight tunnel, the moth was released imme-

diately and observed for 3 min. Behavioral responses, including random and oriented flight, contacts and landings, and oviposition on extract-treated substrates, were recorded. Oriented flight (anemotaxis) was recorded only when it occurred within 65-cm of the substrates in the upwind end of the tunnel where the odor plumes from the two dispensers remained separate. Six replicates of 10–16 (mean = 14) *H. virescens* and *H. subflexa* females were tested individually in each test in all experiments. Tests with untreated controls were conducted periodically to ensure that response to the dispenser substrate (white muslin) was negligible when treated with solvent only and that contamination of the bioassay system had not occurred. The data were converted to arcsin $\sqrt{X}$ and analyzed using ANOVA (Steel and Torrie, 1960). Treatment means were separated using Duncan's multiple-range test (Duncan, 1955).

Four experiments were conducted to determine if: (1) *H. virescens* and *H. subflexa* moths demonstrated significant preferences for extracts from their respective host plants in the flight tunnel, (2) *H. virescens* showed any significant response to extract from resistant tobacco, (3) *H. virescens* responded similarly to extracts from two cultivated host plants, and (4) responses by *H. virescens* to extracts from cultivated and wild host plants were similar in the flight tunnel.

Experiment 1. Behavioral responses and preferences by *H. virescens* and *H. subflexa* females for host and nonhost volatiles were evaluated in dual-choice tests (1–8) using extract from groundcherry leaves and either cotton square extract or extract from cotton, susceptible tobacco, or *Desmodium* leaves.

Experiment 2. In the second experiment, extracts of leaves from susceptible tobacco and the resistant tobacco cultivar were evaluated in choice tests for attracting *H. virescens* females to treated substrates in the flight tunnel.

Experiment 3. Extracts from susceptible tobacco and cotton leaves were used in the third experiment to determine if *H. virescens* showed a preference in the flight tunnel for volatiles from either of these cultivated host plants.

Experiment 4. In the last experiment, *H. virescens* moths were given a choice of extracts of leaves from a cultivated host plant, susceptible tobacco, and a wild host plant, *Desmodium*. Flight and ovipositional responses were observed.

RESULTS AND DISCUSSION

The behavioral responses demonstrated by *H. virescens* and *H. subflexa* females in these dual choice tests were similar to the responses that Tingle et al. (1989) reported when they observed the flight response of *H. subflexa* to an attractant from groundcherry in the flight tunnel. However, in the present study the moths were allowed to demonstrate a preference for either of two plant

extracts that were presented simultaneously on separate substrates in each test. During random (undirected) flight, the moths that began to respond positively with oriented flight to one of the extracts followed the odor trail (plume) toward the extract dispenser. The moths that landed remained on the extract-treated substrate or returned to random flight. Landings on the substrate often were accompanied with oviposition. After returning to random flight, some of the moths responded again to the same extract or responded similarly to the other extract that was being dispensed simultaneously into the flight tunnel. Those moths that did not contact or land on a substrate after oriented flight returned to random flight and may or may not have shown any additional positive response to either extract.

In the dual choice tests (1–4) in experiment 1, mated female *H. subflexa* moths demonstrated a significant preference for extract from their only known host, groundcherry (Figure 1A). Of the moths that responded to either or both of the extracts in each test, 49–85% flew only to the substrate treated with groundcherry extract, whereas 0–14% went only to the substrate treated with a nonhost extract. The percentages of moths that contacted, landed, or landed and oviposited on the extract-treated substrates were consistent with the flight response data (Table 1). The significantly lower response to extracts from cotton leaves and squares and from leaves of susceptible tobacco and *Desmodium* is consistent with the monophagous habit of *H. subflexa*. These data support the results reported by Mitchell et al. (1990) on the oviposition response by *Heliothis* species to plant allelochemicals using the olfactometer and techniques of Mitchell and Heath (1987).

H. virescens, a polyphagous species, responded positively in tests 5–8 (experiment 1) to extracts from susceptible tobacco, cotton, *D. tortuosum* (host plants), and groundcherry, a nonhost (Figure 1B). However, no significant differences between flight responses to groundcherry extract and either of the other plant extracts were demonstrated by the responding moths, with 22–35% orienting toward only one of the extract-treated substrates in a test. At least 42% of the moths flew to both extracts in each test. The contact, landing, and oviposition data were consistent with the flight response data, except that *H. virescens* oviposited significantly more often on the substrate treated with cotton square extract than the groundcherry-treated substrate (Table 1). There is no evidence that *H. virescens* oviposits on groundcherry in nature. Although some eggs were deposited on substrates treated with nonhost extracts, Laster et al. (1982) reported that *H. virescens* were unable to complete development on groundcherry. Similarly, *H. subflexa* larvae rejected cotton, and only one individual survived on tobacco in their study.

When given a choice of volatiles from extracts of leaves from susceptible tobacco and a resistant cultivar in experiment 2, *H. virescens* females showed a significant preference for susceptible tobacco extract (Figure 2). Significantly

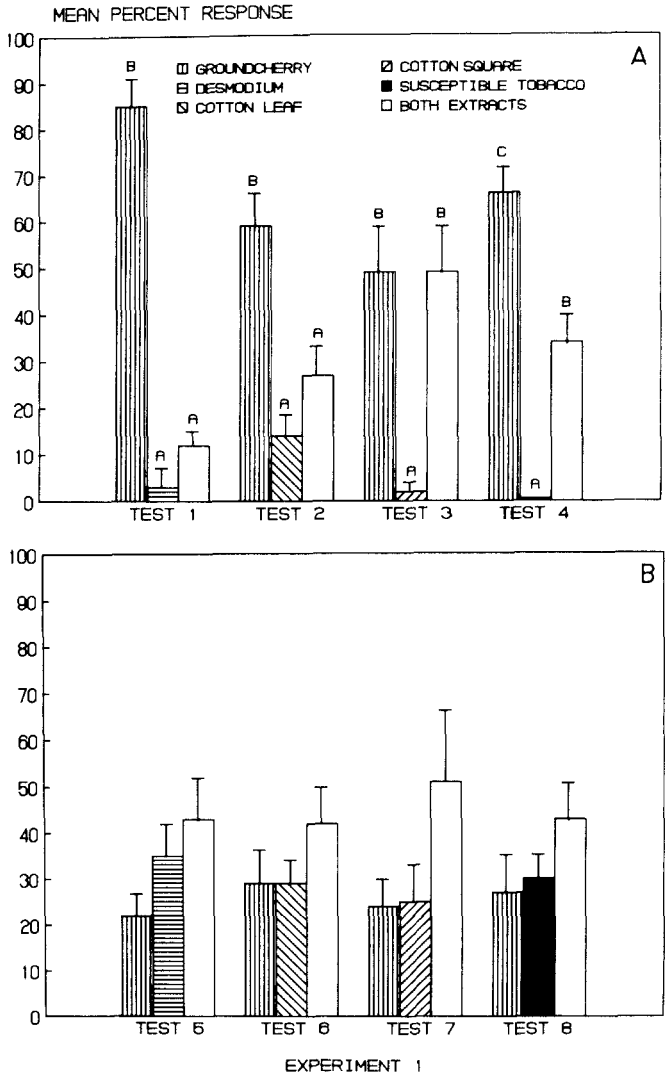


FIG. 1. Flight response of *Heliothis subflexa* (A) and *H. virescens* (B) females exposed to plant extracts. Mean percentages indicate positive anemotactic response (i.e., oriented flight toward the odor source) of moths to either or both extracts in each test; means within each test with the same letter are not significantly different, $P < 0.05$ [ANOVA (Steel and Torrie, 1960) and Duncan's multiple-range test (Duncan, 1955)]. Bars on top of columns indicate standard error of means.

TABLE 1. RESPONSE OF *Heliothis subflexa* (HS) AND *H. virescens* (HV) FEMALES TO HOST AND NONHOST PLANT EXTRACTS IN FLIGHT TUNNEL, EXPERIMENT 1

Test number[a]		Mean % ± SE responding[b]			Mean % ± SE of responding moths that contacted or landed on each treated substrate[c]			
					HS		HV	
HS	HV	HS	HV	Plant extract	Contacted[d]	Oviposited	Contacted[d]	Oviposited
1(80)	5(86)	58 ± 5.2	66 ± 4.3	Groundcherry	73 ± 8.7b	47 ± 7.4b	21 ± 4.4a	20 ± 6.7a
				Desmodium	2 ± 2.0a	0 ± 0a	26 ± 6.5a	24 ± 4.5a
				Both extracts	3 ± 3.0a	2 ± 1.7a	26 ± 8.4a	9 ± 2.2a
2(90)	6(85)	48 ± 5.9	55 ± 4.6	Groundcherry	57 ± 7.1b	56 ± 8.9b	24 ± 6.4a	15 ± 5.3a
				Cotton leaf	14 ± 3.3a	14 ± 6.7a	29 ± 2.4a	21 ± 3.5a
				Both extracts	17 ± 6.4a	5 ± 3.4a	30 ± 6.4a	18 ± 4.1a
3(73)	7(84)	52 ± 6.1	45 ± 5.1	Groundcherry	35 ± 8.5b	29 ± 8.6b	12 ± 3.8a	3 ± 2.8a
				Cotton Square	2 ± 1.9a	2 ± 2.0a	25 ± 4.9a	25 ± 9.5b
				Both extracts	30 ± 9.9b	16 ± 8.2b	34 ± 9.9a	9 ± 4.8ab
4(84)	8(80)	59 ± 6.8	48 ± 4.1	Groundcherry	58 ± 7.6c	55 ± 6.3c	11 ± 3.5a	5 ± 3.3a
				Tobacco[e]	0 ± 0a	0 ± 0a	17 ± 6.8a	15 ± 3.9a
				Both extracts	20 ± 5.0b	8 ± 3.6b	24 ± 3.6b	10 ± 4.9a

[a] Six replications per test per species; each replication consisted of 10–16 moths flown individually. The value in parenthesis indicates the total number of moths in each test.

[b] Includes moths demonstrating a positive anemotactic response (i.e., oriented flight within 65 cm of the odor source) to either or both extracts in each test.

[c] Percentages in columns in each test followed by the same letter are not significantly different, $P < 0.05$ [ANOVA (Steel and Torrie, 1960) and Duncan's multiple range test (Duncan, 1955)].

[d] Means of 83 ± 3.6 and 85 ± 2.5% of the HS and HV, respectively, that contacted a substrate treated with either a host or nonhost extract landed on that substrate.

[e] Susceptible tobacco.

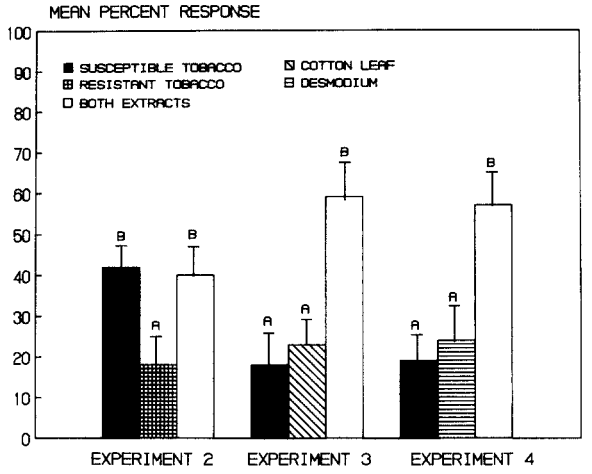


FIG. 2. Flight response of *Heliothis virescens* females exposed simultaneously to extracts from susceptible tobacco and either resistant tobacco (experiment 2), cotton (experiment 3), or *Desmodium* (experiment 4). Means with the same letter in each experiment are not significantly different, $P < 0.05$ [ANOVA (Steel and Torrie, 1960) and Duncan's multiple-range test (Duncan, 1955)]. Bars on top of columns indicate standard error of means.

fewer moths demonstrated a positive flight response to resistant tobacco extract than to extract from susceptible tobacco leaves. More than half of the 42% that flew only to susceptible tobacco extract landed and oviposited on the treated substrate, whereas one-third of the 18% that oriented only to the substrate treated with resistant tobacco extract landed and deposited eggs.

H. virescens moths showed no preference between extracts from susceptible tobacco and either cotton (experiment 3) or *Desmodium* (experiment 4) in the flight tunnel (Figure 2). Approximately 58% of the responding moths demonstrated positive anemotaxis to both extract-treated substrates in experiments 3 and 4. There were no significant differences in the number of contacts and landings or oviposition on the extract-treated substrates. More than three-fourths of the *H. virescens* that responded to any of the extracts in these experiments contacted or landed on the substrates, and most of these moths deposited eggs.

In the "choice" bioassays conducted by Mitchell et al. (1990), in which they used an oviposition olfactometer (Mitchell and Heath, 1987), significantly more *H. virescens* eggs were deposited on the substrate treated with tobacco extract than those substrates treated with extract from either cotton or *Desmodium* leaves. However, since continuous observations were not made during oviposition, it was not determined what percentage of moths deposited eggs on

both substrates. Although *Heliothis* females may be attracted to a particular plant species, cues that trigger oviposition may not necessarily be present when contact is made with the plant's surface.

The results presented here demonstrate clearly that host location by *H. virescens* and the sibling species, *H. subflexa*, is mediated by volatile plant constituents. It also is evident that some of the plant extracts tested contained chemicals that induced females to oviposit upon contact with the treated cloth substrate. It is yet unclear, however, whether the chemicals that induced anemotaxis are the same compounds that stimulated oviposition. Nevertheless, if the chemicals necessary for attraction are lacking, then the probability of a pest such as *H. virescens* becoming established on a particular plant species would be greatly diminished even if the plant had chemicals that might otherwise stimulate females to oviposit. Thus, the flight-tunnel bioassay could become a valuable adjunct to methods currently used to screen and select germ plasm that might confer a degree of resistance to attack by *H. virescens*.

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SEDUCIN, MALE SEX PHEROMONE OF THE COCKROACH *Nauphoeta cinerea*: ISOLATION, IDENTIFICATION, AND BIOASSAY

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Abstract—The male sex pheromone of the cockroach *Nauphoeta cinerea* was isolated from adult sternal glands and identified by gas chromatography–mass spectrometry. This pheromone, which attracts females from a distance, is a multicomponent blend. It is composed principally of three compounds: 3-hydroxy-2-butanone (acetoin), 2-methylthiazolidine, and 4-ethyl-2-methoxyphenol. A mixture of synthetic compounds in a ratio of 4 : 4 : 1 (2700 ng) elicits maximal response and short latency of response by receptive females, similar to that induced by male sternal gland extracts. Tested separately, each compound induces a positive response, but the moving times spent by females are generally longer than with the mixture or extracts. These three volatile compounds also are found in small amounts (about $\frac{1}{40}$ of sternal glands) in tergal gland secretions licked by females. Acetoin also is found in the sternal and tergal glands of two closely related species *Leucophaea maderae* and *Henschoutedenia flexivitta*. The biological relationship of each compound and pheromone blend to behavioral response is discussed.

Key Words—Acetoin, 3-hydroxy-2-butanone, 2-methylthiazolidine, 4-ethyl-2-methoxyphenol, 4-ethylguaiacol, male sex pheromone, pheromone blend, cockroach, *Nauphoeta*, Blattaria, Dictyoptera.

INTRODUCTION

Among cockroaches, chemical signals (pheromones) were shown to play an important role, especially in the regulation of sexual behavior (Roth and Willis, 1952, 1954; Roth and Barth, 1967; Barth, 1968), but so far only a few sex

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pheromones have been elucidated and confirmed by synthesis. The pheromones identified play different roles in the behavioral, courtship, and mating sequences. In many cockroach species, the sexual behavior can be summarized as follows (Sreng, 1984): a "sex pheromone" emitted by one of the sexual partners attracts individuals of the opposite sex from a distance (Figure 1a). Once the sexes meet, the male recognizes the receptive female by contact chemoreception upon antennal fencing (Figure 1b) and raises his wings (Figure 1c) caused by "wing raising pheromone" or "female contact sex pheromone." The receptive female is attracted to the male's back, mounts, and feeds on the male's tergal gland secretion or "aphrodisiac sex pheromone" (Figure 1d). This behavior places the female in the proper mating position and serves as a stimulus for the male to copulate (Figure 1e).

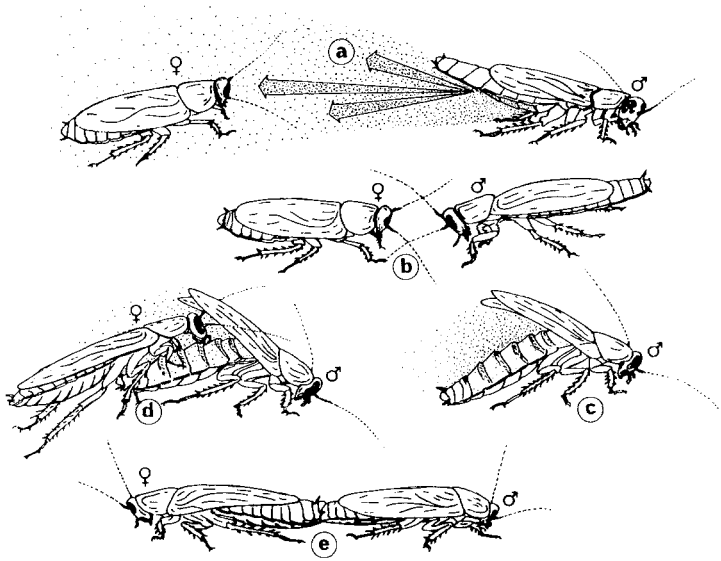


FIG. 1. Different fundamental sequences of sexual behavior (type A behavior pattern) of cockroach, especially in *Nauphoeta cinerea* (Sreng, 1983). The male adopts a characteristic calling behavior (a). His abdomen raises and flexes upwards, exposing the sternal glands. In this posture, the male releases "sex pheromone" that attracts the female from a distance. When the attracted female is in close contact, the male recognizes her by antennal contact chemoreception (role of "Wing-raising pheromone") (b) that incites the male to raise his wings (c). In male wing-raising posture, the tergal glands are exposed and attract the female. She mounts the male's back and feeds on the tergal gland secretion (d) or "aphrodisiac sex pheromone," which is necessary for the male to realize copulation. The male pushes his abdomen under the female to make a connection with her genitalia and the pair in copulation assume the typical false-linear position (e).

At present, there are about 4000 known cockroach species in the world, but only a few chemical signals are well known: the female sex pheromone component of *Periplaneta americana* (Persoons et al., 1979, 1990; Hauptmann et al., 1986; Biendl et al., 1989) and of *P. japonica* (Takegawa and Takahashi, 1989), the wing-raising pheromone of *Blattella germanica* (Nishida et al., 1974; Nishida and Fukami, 1983) and of *Nauphoeta cinerea* (Takahashi and Fukui, 1980, 1983).

In *Nauphoeta cinerea*, the male produces two main chemical signals: (1) a sex pheromone called "seducin" by Roth and Dateo (1966), which originates in the abdomen (Roth and Dateo, 1966; Barth, 1968; Dimeo et al., 1978) and attracts the female from a distance, and (2) an aphrodisiac sex pheromone that attracts the female to the male's back to feed on the tergal secretions. *N. cinerea* males possess complex abdominal exocrine glands (tergal and sternal glands), and in mating behavior, the male adopts a "calling posture" (Sreng, 1979b, 1983), identical to the "aggressive posture" observed by Ewing (1967). In this behavior the male exposes the sternal glands, releasing the sex pheromone that attracts the female from a distance.

The three main compounds that constitute the male sex pheromone of *N. cinerea*, seducin, 3-hydroxy-2-butanone (acetoin); 2-methylthiazolidine; and 4-ethyl-2-methoxyphenol, were first tentatively identified by Sreng (1983), but due to the lack of some synthetic compounds, their exact structures and pheromone blend were not confirmed. In this paper, we report the isolation, identification, and bioassay of the natural and synthetic compounds (separately and in combination) of the sex pheromone blend seducin secreted by male sternal glands, and the bioassay techniques in measuring moving times spent by females, to evaluate the quality of different stimuli. The natural and synthetic compounds are compared.

METHODS AND MATERIALS

Insect. Colonies of cockroaches *N. cinerea* were reared in glass aquaria (60 × 60 × 20 cm) and maintained at 25–27°C under a 12 : 12 dark–light cycle, 70% relative humidity with access to dry dog food and water. Last-instar male and female nymphs were isolated, and the date of adult ecdysis was recorded. The males and females were kept in separate plastic boxes (9 × 6 × 2 cm) in groups (3 insects/box) until used (males for pheromone gland extracts, females for behavioral tests).

Preparation of Pheromone Gland Extracts. Male adults of *N. cinerea* produce their sex pheromone from a well-developed sternal gland localized on abdominal sternites 3–7 (Sreng, 1979b, 1983, 1984). Males (9–10 days old) were anesthetized under CO_2 gas for 20 sec, and the sternal gland was dissected

with microscissors and forceps. The gland was removed from adjacent tissues (hemolymph, fat body) with a paper towel and transferred to a vial of distilled methylene chloride (100 μl/sternal gland) and allowed to soak for 2 hr, then the cuticular gland was removed from the solvent. Individual gland extracts (for GC quantitation of pheromone component) and a pooled sample of 50 sternal glands (for GC-MS analysis or bioassay) were prepared. The extracts were then evaporated to a small volume under a nitrogen stream or held in a freezer at −20°C prior to use. The same procedure was used to extract male tergal glands (tergites 2–8) or female cuticular sternites for bioassay tests.

GC and GC-MS Analysis. The analysis of sex pheromone extracts was performed on a Girdel series 300 gas chromatograph using a capillary column (30 m × 0.30 mm ID), Supelcowax 10 column, bonded Carbowax PEG 20 (Supelco France), or a packed column (3 m × 5 mm ID, Carbowax 20 M 5%). The GC with a splitless sample injector and a flame ionization detector was connected with an integrator (Shimadzu CR-3). The temperature was programmed from 55°C (initial time 5 min) to 180°C at 5°C/min (final time 40 min). To analyze an individual gland, quantitation of the sex pheromone component (3-hydroxy-2-butanone, 2-methylthiazolidine, 4-ethyl-2-methoxyphenol) was carried out in a packed column by adding 100 ng of (*Z*)-11-tetradecen-1-ol acetate (*Z*11–14:OAc) to each extract. With this system, peak resolution was adequate to calculate the quantity and proportion of each component. Identification of sex pheromone components was based on external standards of retention times from compounds identified by GC-MS.

The biologically active extracts (pooled sample of 50 sternal glands) and authentic compounds were identified by EI (70 eV) GC-MS, using a capillary column as above in a Nermag R10-10C mass spectrometer. Acquisition and processing of mass spectra were set by a connected computer (Digital Equipment Corp.), and identification of each compound was made automatically, referring to a spectrum library.

Chemicals. Z11–14:OAc, 3-hydroxy-2-butanone (acetoin), and 4-ethyl-2-methoxyphenol were purchased from Interchim, (Montluçon, France). The 2-methylthiazolidine was generously synthesized by Dr. A. Tabard, Laboratoire de Chimie des Hétérocycles, CNRS UA 33, Université de Bourgogne, France, according to the procedure of Sheehan et al (1956). All chemicals were >98% pure as determined by capillary GC analysis and were used without further purification.

Bioassay Procedures. Virgin female cockroaches (9–12 days old) were used to test the olfactory stimuli in an olfactometer. A Y-maze olfactometer was constructed from glass tube (ID 2 cm) that consisted of a common arm (30 cm), with a 80° angle between two choice arms (20 cm each). A start point, A (in the common arm), and two arrival points, B and C (in the choice arms), were marked 3 cm from the ends of each arm. To test the samples, the filter papers impregnated with the stimuli source were placed between arrival points

B and C and the ends of each arm, where the air current arrived. Air current speed through each of the choice arms, measured with soap bubbles, was 2 cm/sec and was 4 cm/sec through the common arm. The samples (extracts or synthetic compounds) were applied onto 1-cm^2 filter paper squares, and then the pieces of filter paper were sandwiched between wire fencing, preventing the females from carrying the papers away.

To test the odor stimuli, the amounts of stimuli for optimum response were determined after preliminary tests. The quantities of 1, 0.5, and 0.25 male sternal gland equivalents; one male tergal gland equivalent; one female cuticular sternal extract equivalent; 1200 ng of 3-hydroxy-2-butanone and of 2-methylthiazolidine; 300 ng of 4-ethyl-2-methoxyphenol separately or in a mixture (2700 ng) (ratio 4:4:1) were used. Female responses were less than 50% when tested separately with 600 ng of 3-hydroxy-2-butanone or 2-methylthiazolidine or with 200 ng of 4-ethylguaiacol. Virgin females were given the choice between the odor stimuli (extracts or chemicals) and the control (solvent, CH_2Cl_2). The pieces of filter paper impregnated with the odor stimuli or solvent were changed after every five individual tests. All tests were conducted in the dark room, under photographic red light between the end of the sixth and beginning of the tenth hour into the scotophase. Ten individual virgin females were used for each test. All individual females were placed in a adaptation chamber (6 × 9 × 5 cm) at least 2 min before each test, with a wire fencing screen door that could be raised to allow entry into the common arm at the start point A. The females were allowed to run down the cylinder, turning either right or left upon reaching arrival points B or C. For control experiments, each female was first allowed to move through the olfactometer from start point A to arrival points B or C in the presence only of the clean airstream (first passage). The time taken to move from A to B or C (in seconds) and the turning choices (right or left) were recorded. In the second phase (second passage), the location of odor stimulus position (right or left) was randomized with respect to the first turning of individual females (to compensate for the turning tendency phenomenon). Each assay was run for a maximum of 5 min from the starting point A. If a female was attracted to an odor stimulus side in less than 5 min, she was considered to give a positive response, and the moving time from A to B or C was recorded and compared with the control (clean airstream), otherwise she was considered to give a negative response. The olfactometer was cleaned with 90% ethanol to prevent contamination by pheromones or chemicals.

RESULTS

Chemical Analysis of Pheromone Secretion. GC analysis of the volatile sex pheromone compounds of male sternal gland extracts (St3–St7) in CH_2Cl_2 (1 male equivalent, 9–10 days old) from pooled gland extracts is shown in Figure

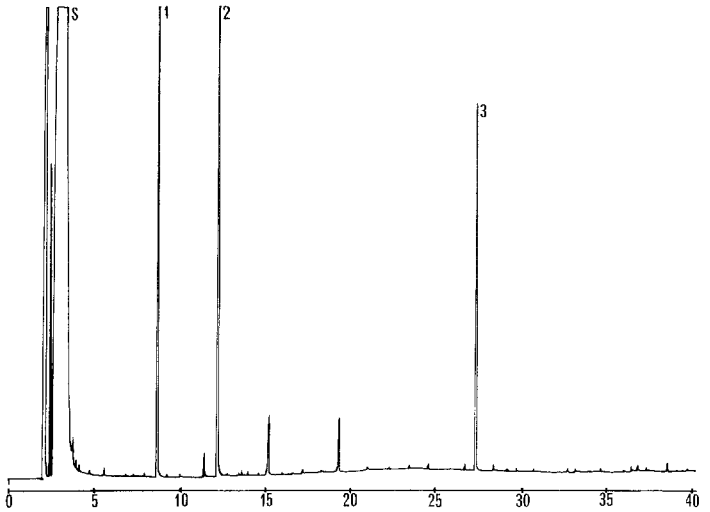


FIG. 2. A typical gas chromatogram of male sex pheromone of *Nauphoeta cinerea*, obtained from analysis of male sternal glands (1 male gland equivalent, 9–10 days old) using Supelcowax 10 capillary column. Three peaks are identified: (1): 3-hydroxy-2-butanone (acetoin); (2): 2-methylthiazolidine; (3): 4-ethyl-2-methoxyphenol (4-ethylguaiacol). The temperature was programmed from 55°C (5 min initial time) to 180°C at 5°C/min (40 min, final time). S = solvent.

2. The chromatogram can be considered typical, revealing the presence of three major peaks (1, 2, 3) and three minor peaks. Only the three major compounds were identified and tested.

Peak 1 (m/e = 88, Figure 3) was identified as 3-hydroxy-2-butanone (others names: acetoin, acetyl methyl carbinol and dimethyl ketol in The Merck

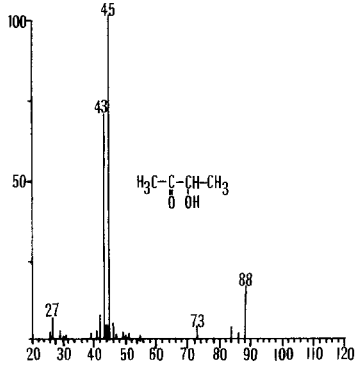


FIG. 3. El mass spectrum of natural compound of 3-hydroxy-2-butanone (acetoin).

Index, 1976). The retention time and mass spectrum of the authentic sample were identical to those of the natural compound.

Peak 2 (m/e = 103, Figure 4) was identified as 2-methylthiazolidine by comparison of retention time and GC-MS of synthetic compound and natural substance.

Peak 3 (m/e = 152, Figure 5) was identified as 4-ethyl-2-methoxyphenol (4-ethylguaiacol). Retention time and mass spectrum of the authentic sample and natural compound were identical.

GC analysis of extracts of individual male sternal glands, just after emergence (less than 3 hr) to 1–2 days old, indicates that they did not contain detectable amounts of any of the three compounds. However, in sternal gland extracts of 3 to 4-day-old males, detectable amounts of these three compounds were present in all the individuals, but the concentration was low. Quantitation of

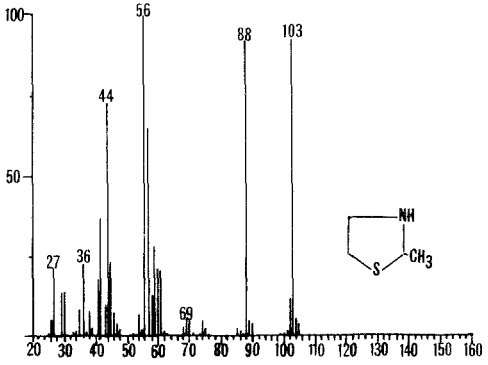


FIG. 4. El mass spectrum of natural 2-methylthiazolidine.

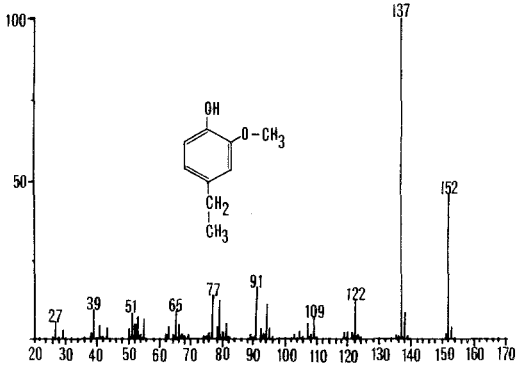


FIG. 5. El mass spectrum of natural 4-ethyl-2-methoxyphenol (4-ethylguaiacol).

the three major compounds identified in mature virgin males (9–10 days old) are given in Table 1. The amount and ratio of each compound in sex pheromone glands vary widely between individual insects. From the analysis of 10 individual sternal glands, the amount and ratio range from 1000 ng to 3000 ng, 45–75% for 3-hydroxy-2-butanone, 400–2500 ng, 25–50% for 2-methylthiazolidine, and 50–400 ng, 3–12% for 4-ethyl-2-methoxyphenol.

GC analysis of tergal gland extracts, licked by females, shows that the three major compounds identified in sternal gland (acetoin, 2-methylthiazolidine, 4-ethylguaiacol), are present (ratio 3 : 2 : 1) in a small amount (about $\frac{1}{40}$ of sternal glands).

Bioassay. The biological activities of male sternal and tergal gland extracts, female cuticular sternal extracts, synthetic compounds, 3-hydroxy-2-butanone, 2-methylthiazolidine, 4-ethyl-2-methoxyphenol (separately or in a mixture), and moving times (first and second passages), are shown in Table 2. When female sternal cuticular extracts were tested, the responses of females were slower and the moving times were long (occasionally more than 5 min); a female walked slowly down the cylinder, stopped, licked antennae, and sometimes returned to the place of departure before reaching an arrival point. Female sternite extracts did not elicit positive responses. In 10 females tested, six reached arrival points, only two turned to the extracts, and the moving times were long and not significantly different from the first passage. With male sternal gland extracts, receptive females were attracted quickly to the samples and the moving times were short. Their antennae vibrated at a rapid rate, and they ran directly to the source. The doses of 1 and 0.5 male sternal gland extract equivalents induced a slight inhibition of female responses (respectively, 70% and 80%). The highest percentage of female responses (100%) and the lowest moving times spent by females (from A to B or C) were obtained with tests of 0.25 male equivalent (about 900 ng of mixture) of male sternal gland extracts (38 ± 9 sec) and with

TABLE 1. AMOUNT AND PERCENTAGE OF THREE COMPOUNDS IDENTIFIED IN MALE STERNAL GLANDS OF *Neuphoeta cinerea*[a]

	Compound		
	Acetoin (1)	2-Met. thiaz. (2)	4-Et guaiac. (3)
Amount (ng)	1937 ± 236	1439 ± 271	180 ± 34
N	10	10	10
Percent	55	40	5

[a] GC analysis of 10 individual glands (9–10 days old): (1) 3-hydroxy-2-butanone (acetoin), (2) 2-methylthiazolidine (2-Met.thiaz), (3) 4-ethyl-2-methoxyphenol (4-Et. guaiac.). The data are the means of 10 measures ± SEM expressed in nanograms of pheromone produced per male.

TABLE 2. MOVING TIMES AND FEMALE RESPONSES TO DIFFERENT STIMULI IN Y-MAZE OLFACTOMETER

First passage		Second passage			
Contr.	Mov. times (sec)[a]	Stimuli	Mov. times (sec)	Female responses (%)	*t* tests (*P*)
1 Air	142 ± 20 a	Air	151 ± 21 a		>0.5
2 Air	177 ± 20 a	Fem. Cu. Ext.	165 ± 65 a	+20	>0.5
3 Air	163 ± 17 a	M. St. Ext. 1	78 ± 8 b	+70	<0.05
4 Air	141 ± 12 a	M. St. Ext. 0.5	81 ± 14 b	+80	<0.01
5 Air	97 ± 9 a	M. St. Ext. 0.25	38 ± 9 b	+100	<0.001
6 Air	134 ± 13 a	M. Terg. Ext. 1	79 ± 17 b	+80	<0.02
7 Air	214 ± 19 a	Mixt. 1, 2, 3	69 ± 10 b	+100	<0.001
8 Air	158 ± 18 a	Acetoin	90 ± 10 b	+90	<0.01
9 Air	187 ± 18 a	2-Met. thiaz.	114 ± 15 b	+80	<0.01
10 Air	211 ± 17 a	4-Et. guaiac.	171 ± 14 a	+70	>0.05

[a] The moving times spent by female during a control test in the airstream (first passage) are compared to those of the tests with stimuli (chemical or extract), (second passage). Data of the moving times are the mean of 10 measurements ± SEM spent by female expressed in seconds. Means followed by the same letter in the same line are not significantly different (Student's *t* test). Mov. times = moving times; Contr. = control; Fem. Cu. Ext. = female cuticular sternal extract (1 female equivalent); M. St. Ext. 1, 0.5, 0.25 = male sternal gland extract (1, 0.5 and 0.25 male equivalents); M. Terg. Ext. 1 = male tergal gland extract (1 male equivalent); Mixt. 1, 2, 3 = mixture of synthetic compounds: (1) acetoin, (2) 2-methylthiazolidine, (3) 4-ethylguaiacol in a ratio 4:4:1 (2700 ng); acetoin = 3-hydroxy-2-butanone (1200 ng); 2-Met.thiaz. = 2-methylthiazolidine (1200 ng); 4-Et. guaiac. = 4-ethylguaiacol (300 ng); + = positive responses.

a mixture of three synthetic compounds (ratio 4:4:1; 2700 ng) (69 ± 10 sec). For the tergal gland extracts, the female responses were positive, but the moving times (79 ± 17 sec) were higher than in sternal gland extracts ($\frac{1}{4}$ equivalent). Each single synthetic compound was tested separately (1200 ng for 3-hydroxy-2-butanone and 2-methylthiazolidine; 300 ng for 4-ethylguaiacol). The data show that all these three compounds were less active when tested separately and the moving times of the females were generally longer than with the mixture. The moving times spent by females with all active samples are shorter (second passage) than in the control (airstream, first passage).

DISCUSSION

This is the first male sex pheromone identified in cockroaches that attracts females from a distance. Components of the female sex pheromone blend of *P. americana* have been identified by Persoons et al. (1979, 1990), Hauptmann et al. (1986), and Biendl et al. (1989). The name seducin, for the male sex pher-

omone isolated from the abdomen of *N. cinerea* was first proposed by Roth and Dateo, (1966), but the exact origin of this pheromone, the male sternal and tergal glands, was elucidated 13 years later (Sreng, 1979b). Our results show that seducin is a multicomponent sex pheromone composed of two major compounds, 3-hydroxy-2-butanone and 2-methylthiazolidine (each more than 40%), and a minor compound, 4-ethyl-2-methoxyphenol (less than 10%). The blend of these three compounds represents a stimulus that elicits the maximum response from receptive females, comparable to that observed with male sternal gland extract (0.25 equivalent) but the moving times are higher. This suggests a role of the minor compounds detected but not identified in the pheromone blend of seducin. The mixture of synthetic compounds (ratio 4:4:1) would be very close to the natural pheromone and could be considered as the pheromone blend. A slight inhibition in female responses when tested with 1 or 0.5 male gland extract equivalents may be due to the excessive concentration used.

It is now well established that many insect species, including moths, use multicomponent sex pheromones as species-specific communication signals. In *P. americana*, the attraction of males to calling females is strictly species-specific (Seelinger 1984). In this species, virgin females produce a number of volatile substances that elicit search and sexual behavior in the males (Persoons, 1977), but until recently, only two compounds, periplanone A and B (PA and PB) were studied (Persoons et al., 1976, 1979, 1982; Tobin et al. 1981; Sass, 1983, Seelinger, 1985; Nishino et al., 1983, 1989; Hauptmann et al., 1986). Very recently, Biendl et al. (1989) isolated two more biologically active compounds, namely periplanone D1 and periplanone D2. Persoons et al. (1990) proposed for these two novel components the names periplanone C (PC) and periplanone D (PD), respectively. It would be interesting to know if PA, PB, PC, and PD are really the complete sex pheromone blend of and species-specific to *P. americana* or only the major compounds of the sex pheromone blend in some Blattidae species.

Our data on *N. cinerea* and GC analysis of the sternal glands of the two closely related species, *Leucophaea maderae* and *Henschoutedenia flexivitta*, belonging to the subfamily Oxyhaloinae (McKittrick 1964) and having the same mating behavior pattern, male sex pheromone, and abdominal glands (Sreng, 1983, 1984), show that the male pheromone blends of these species all possess acetoin but not other compounds (Sreng, unpublished data). It is clear now that the male sex pheromone blend of *N. cinerea* is distinguishable from two related species by its different components rather than by different ratios.

Acetoin is a product of fermentation, obtained by the action of sorbose bacterium or fungi. In insect species, acetoin was identified only in the odor bouquets of the males and females of bark beetle (Francke et al., 1988). As far as we know, 2-methylthiazolidine was not identified in other insect species as

an attractant or a sex pheromone. 4-Ethyl-2-methoxyphenol is used as an attractant for the northern corn rootworm (McGovern and Ladd, 1988).

The wide variability in the ratio of the components of the pheromone blend in males of *N. cinerea* seems to be related to the presence of hierarchies between males (Ewing, 1967; Breed et al., 1980; Schal and Bell, 1983) after fighting (dominant–subordinate) in the group. Only the dominant male (winner of combat) adopts the "aggressive posture," whereas the loser adopts "subordinate posture" (Ewing 1967). The results of individual GC analysis suggest that each male has a characteristic ratio of pheromone blend (personal odor) according to his status in the group.

The aggressive posture observed by Ewing (1967) in fighting behavior is identical to the calling posture in mating behavior proposed by Sreng (1979b): the male abdomen is flexed upwards exposing the sternal glands and releasing the sex pheromone (Figure 1a). In this posture, the dominant male attracts receptive females more strongly than subordinate males. This is in agreement with observations of Breed et al. (1980), Schal and Bell (1983), and Moore (1989), who showed that the dominant males copulate more often than subordinate males.

Pheromone production is closely related to gland development and attraction of females. The type 3 cells of glandular epithelium, which are thin and contain no secretions just after imaginal molt, are filled, three days later, with numerous clear vacuolar secretions (Sreng, 1985). This result is in agreement with behavioral studies (Roth and Dateo, 1966; Dimeo et al., 1978). However, pheromone production does not decrease after mating (Roth and Dateo, 1966) and does not seem to be controlled by corpora allata (Hartman and Suda, 1973).

In many cockroach species, the female releases an "attractive sex pheromone" that attracts males from a distance (e.g., *P. americana*), and the male produces an "aphrodisiac sex pheromone" that is licked by the females. In *N. cinerea* and six other closely related species, males produce attractive sex pheromones (sternal glands) and aphrodisiac sex pheromones (tergal glands) (Sreng, 1984). Our data in tergal bioassay tests indicate that the females respond positively to the extracts. It is interesting to find, in tergal gland secretions of *N. cinerea*, the three volatile compounds composing the male sex pheromone, in a low concentration mixed with fatty acids and proteins (Sreng, 1983). These volatile compounds induce the female to mount the male's back and to feed on nonvolatile secretions of tergal glands. These results show clearly that the occurrence of volatile compounds in the aphrodisiac sex pheromones at a low concentration is effective for mounting and feeding behavior and, at high concentration, attracts the female from a distance as a sex pheromone. This is in agreement with biochemical data of tergal glands (Brossut et al., 1975; Sreng, 1983) and, according to ultrastructural investigations (Sreng, 1979a, 1985),

suggests that the tergal glands chiefly produce fatty acids and proteins but that, in addition, volatile compounds are given off in small quantities. It is interesting to note that the tergal secretion is not specific for receptive females since it also attracts males and larvae (Sreng, 1983). This suggests that the tergal secretion acts as a stimulant for receptive females and as an appetizer for other individuals. More information is needed concerning volatile components of many male tergal glands (Roth, 1969) in other species whose females produce sex pheromones.

The common occurrence of male sex pheromones, of male-developed abdominal glands, of male hierarchies, and the occurrence of three different patterns of mating behavior (types A, B and C) in seven closely related cockroach species (Subfamily Oxyhaloinae) (Sreng, 1983, 1984) is striking. In mating type B and C patterns, there is no licking of the male's tergal secretions by the females. However, the biological and behavioral significance of attractive sex pheromones and aphrodisiac sex pheromones in other species remains to be investigated and should provide the clue to understanding the evolutionary relationship between chemical signals, abdominal glands, different patterns of mating behavior, and male hierarchy systems in the group. In cockroach species, the identification of male attractive sex pheromones and aphrodisiac sex pheromones that attract females should have as much practical importance as the identification of female sex pheromones.

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THE PROTECTIVE EFFECT OF ANTIOXIDANTS TO A PHOTOTOXIN-SENSITIVE INSECT HERBIVORE, *Manduca sexta*

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Abstract—Photo-activated plant secondary compounds have been shown to be toxic to many organisms including insects. Insect defenses include behavioral mechanisms such as light avoidance, as well as specific biochemical defenses such as antioxidants and antioxidant enzymes. These antioxidant defenses eliminate or quench the deleterious singlet oxygen and free radicals formed by these phototoxins. In this paper we examined the role of dietary antioxidants in protecting the phototoxin-sensitive insect herbivore *Manduca sexta*. Elevated dietary levels of the lipid-soluble antioxidants β-carotene and vitamin E resulted in a concentration-dependent reduction in the mortality associated with treatment of *M. sexta* larvae with the phototoxic thiophene α-terthienyl. Elevated levels of dietary ascorbic acid had no effect, whereas reduced levels greatly increased the toxicity of α-terthienyl. Tissue levels of antioxidants were shown to increase substantially in larvae fed antioxidant-supplemented diets. The results suggest that the ability to absorb and utilize plant-derived antioxidants could be an important defense against photo-activated plant secondary compounds and may have allowed some insects to exploit phototoxic plants.

Key Words—*Manduca sexta*, Lepidoptera, Sphingidae, tobacco, hornworm, antioxidants, α-terthienyl, ascorbic acid, β-carotene, vitamin E.

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INTRODUCTION

Many plant secondary compounds are capable of acting as photosensitizers and causing biological damage to insects (Towers, 1984; Knox et al., 1987). Upon absorption of light, the photooxidants, which are the sensitizers of interest in this study, generate, either directly or indirectly, highly toxic species of oxygen including singlet oxygen and free radicals such as superoxide and hydroxy anions. Biological damage is caused primarily through the oxidation of proteins, lipids, and nucleic acids (Spikes, 1977).

A large degree of variation in the sensitivity of insect herbivores to these plant phototoxins has been demonstrated (Champagne et al., 1986; Iyengar et al., 1987; Berenbaum and Lee, 1990) and it has been postulated that some insects may have specific behavioral and biochemical defense mechanisms to deal with either the accidental or necessary ingestion of phototoxic plant chemicals (Larson, 1986; Larson and Berenbaum, 1988; Pardini et al., 1989; Sanberg and Berenbaum, 1989). Behavioral mechanisms of defense include the leaf-tying behavior of tortricid caterpillars feeding on phototoxic *Hypericum perforatum* (Sandberg and Berenbaum, 1989) and the light-avoidance behavior of *Chrysolina* larvae (Fields et al., 1990). Recently, much work has focused on the presence of antioxidant enzymes such as superoxide dismutase, catalase, glutathione reductase, and peroxidases in lepidopteran larvae (e.g., Pritsos et al., 1988; Lee and Berenbaum, 1989). In mammalian systems these enzymes are well known to be a primary line of defense against free radical damage (Halliwell and Gutteridge, 1985). In some insects, the levels of these antioxidant enzymes have been shown to increase in response to the ingestion of phototoxins such as the flavonoid quercitin (Pritsos et al., 1988) and the furanocoumarin 8-methoxypsoralen (8-MOP) (Lee and Berenbaum, 1989). These enzymes could provide an important defense mechanism that is complementary to the P-450-based detoxification enzyme system of insects.

There are also a number of low-molecular-weight molecules that are capable of preventing the deleterious effects of free radical and singlet oxygen formation. β-Carotene, ascorbic acid (vitamin C), and α-tocopherol (vitamin E) are all known to be either effective quenchers of toxic oxygen species or to be capable of preventing lipid peroxidation (Halliwell and Gutteridge, 1985). Larson (1986) has reviewed the occurrence of potential inhibitors and quenchers of free radicals and singlet oxygen in insects. These include carotenoids, vitamin E, ascorbic acid, and some amines and sulfur compounds. Uric acid has been postulated to be an important antioxidant in mammals (Ames et al., 1981), and it could have an antioxidant role in insects as well. Little experimental work has been carried out, however, to determine whether these molecules are an effective defense against plant phototoxins. In housefly larvae, Robinson and Beatson (1985) have shown that dietary β-carotene exerts a protective effect in

larvae treated with the photosensitizing dye erythrosine B. Ascorbate, tocopherol, and commercial antioxidants such as butylated hydroxytoluene (BHT) actually enhanced the toxic effect of the dye. In herbivorous insects it is unknown whether dietary antioxidants play a role in protecting them against photooxidative damage.

We have been examining the phototoxin α-terthienyl (α-T), which is a thiophene found in many species of the family Asteraceae and which shows a wide degree of variation in its toxicity to insect herbivores. Larvae of the tobacco hornworm, *Manduca sexta*, are particularly sensitive to this compound (Downum et al., 1984; Downum, 1986; Iyengar et al., 1987), and we have investigated whether any degree of protection can be afforded to this insect by the addition of dietary antioxidants such as ascorbate, β-carotene, and vitamin E.

METHODS AND MATERIALS

Insects and Dietary Antioxidants. A colony of *M. sexta* was raised according to the methods of Bell and Joachim (1976) in an incubator set at 25 ± 1°C, with a 16:8 light–dark photoperiod. Newly hatched larvae were transferred to trays of diet cubes that contained varying amounts of antioxidants. For groups of larvae raised on diets supplemented with β-carotene or vitamin E (0.001, 0.01, 1.0% of diet), the antioxidant was first mixed with 2 ml of linseed oil and then added to 100 g of diet. Control diets also received 2 ml of linseed oil. For diets in which the level of ascorbic acid was altered (0.0024, 0.024, 0.24, 1.2% of diet), the antioxidant was added as a dry ingredient to cooled diet.

Mortality Assessments. Four to seven groups of 10, third-instar larvae (50–80 mg mean weight), which had been raised on diets containing antioxidants, were placed into glass Petri dishes and then treated with α-terthienyl using a Potter's spray tower. After treatment, the larvae, together with diets, were placed under a bank of near-UV lamps (300–400 nm, Westinghouse BLB, 5 W/m^2) for 8 h and then returned to the incubator. Mortality assessments were made at 24 and 48 h after spraying the insects. Five milliliters of a 1.5 mg/ml solution of α-terthienyl in acetone containing 1% propylene glycol was found to result in approximately a 50% mortality in control groups and so this level of treatment was used in all trials. Three round glass cover slips (22 mm diam.) also were included in each Petri dish in order to measure actual application rates. The α-T on the cover slips was dissolved in 1 ml of methanol and its concentration measured using a spectrophotometer (Varian 2200). The application rate was 1.31 ± 0.37 $\mu g/cm^2$.

Tissue Analysis. Vitamin E content was examined in midgut, fat body, and carcass (tissue remaining after removal of digestive tract) tissues of *M. sexta*

using a modification of the procedures of Zaspel and Csallany (1983) and Miller and Yang (1985). Fifty to 100 mg of tissue was dissected into cold 0.15 M NaCl containing 5% ascorbic acid (BDH Chemicals) and then homogenized on ice in 1 ml of 1:1 methanol–5% ascorbic acid in water. Samples then were transferred to 1.5-ml Eppendorf plastic tubes and 0.5 ml hexane added. After vortexing for 10 sec, the tubes were centrifuged in the cold for 2 min in a Beckman microfuge (13,000 g), the hexane layer removed to a glass vial, and the extraction procedure repeated twice. The combined hexane extracts were dried under nitrogen in dim light, reconstituted in 300 μl methanol, and then 20-μl injections used in the HPLC. Unused portions of samples were frozen (−70°C) for subsequent β-carotene analysis.

Vitamin E was analyzed by HPLC with a Perkin-Elmer LC-250 binary pump and an LS-40 fluorescent detector. Vitamin E was detected using an excitation wavelength of 295 nm and emission wavelength of 340 nm. The column used was a Beckman Ultrasphere C18 (4.6 mm × 25 cm) with a solvent system consisting of methanol–water (98:2) delivered at 2.0 ml/min. The elution time of vitamin E was 7.9 min. Extraction efficiencies using vitamin E-dosed samples averaged 87%. Standards were prepared from fresh stock solutions of α-tocopherol (Sigma) in methanol.

β-Carotene was analyzed using a Waters model 441 absorption detector containing a mercury lamp and 436-nm filter. The column was eluted isocratically with methanol–acetonitrile–chloroform (25:60:15) at a flow rate of 1.5 ml/min (Miller and Yang, 1985). Standards were prepared using a stock solution (2 mg/ml) of β-carotene (Sigma) in chloroform. Extraction efficiencies averaged 84% with dosed samples while the retention time of β-carotene was 15.1 min.

RESULTS

Protective Effect of Dietary Antioxidants. The 24-h mortality of α-T treated (1.37 μg/cm^2) groups of larvae fed control diets ranged from 32 to 56%, whereas the 48-h mortalities of these groups ranged from 54 to 84% (Figure 1a). Because of the high level of ascorbic acid in control diets (Figure 1a), we primarily examined the effect of reducing the concentration of this vitamin in the diet on the sensitivity of *M. sexta* to α-T. As the level of the vitamin was reduced (from 0.24% to 0.024%), greater mortality was observed. In contrast, a fivefold increase in the vitamin, from 0.24 to 1.2%, afforded no degree of protection against the phototoxin.

The lipid-soluble antioxidants, β-carotene and vitamin E, were both found to provide excellent protection against the toxicity of α-T. β-carotene, at a con-

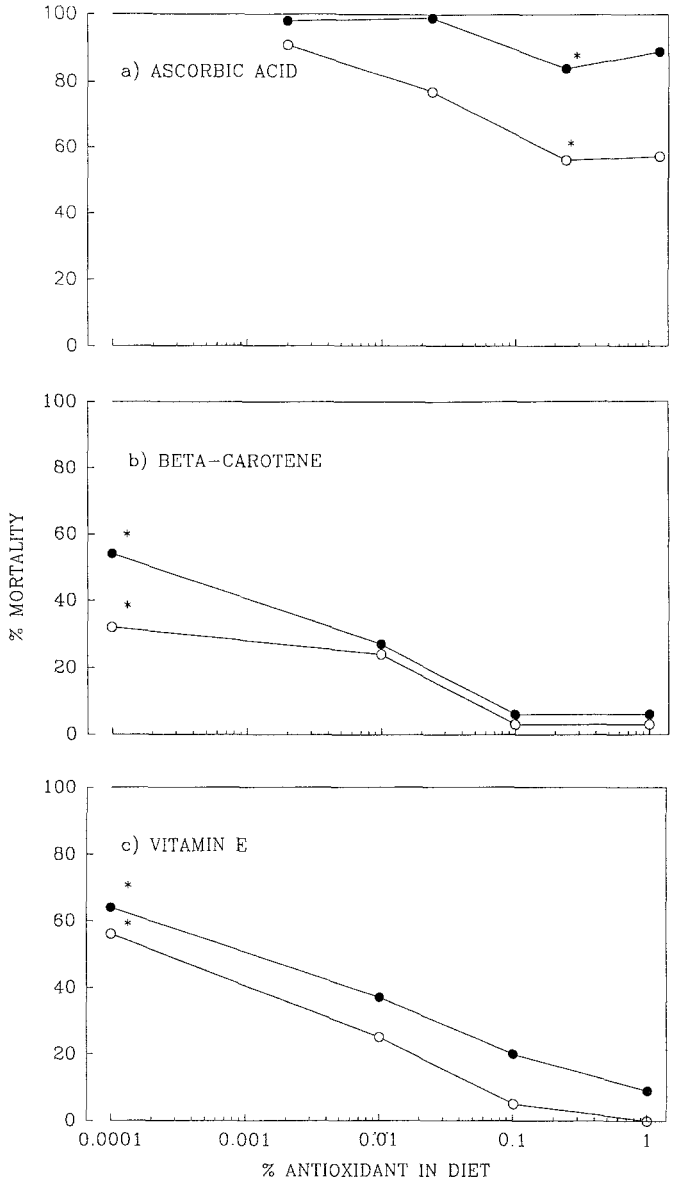


FIG. 1. Toxicity of α-terthienyl to third-instar larvae of *Manduca sexta* raised on diets containing various levels of antioxidants: (a) ascorbic acid, (b) β-carotene, (c) vitamin E. Open circles: 24-h mortality; closed circles: 48-h mortality. Asterisks indicate levels of antioxidant in control diets.

centration of 0.1%, reduced the 24-h mortality of *M. sexta* to only 3% (Figure 1b). Substantial reduction in mortality (to 24%) also was seen at a 10-fold lower level of this antioxidant (0.01%), whereas 10-fold greater levels (1.0%) provided no further protection. The 48-h mortality values closely reflected the 24-h trends with only slightly greater mortality at each level of antioxidant.

The results with vitamin E (Figure 1c) resembled those of β-carotene. A significant level of protection (mortality dropped to 25%) was observed at the 0.01% level while near maximal protection (mortality only 5%) was obtained using 0.1% vitamin E. The 48-h mortalities again only showed slight increases at each level of antioxidant.

Tissue Analysis. In order to confirm that the lipid-soluble antioxidants that had provided protection against α-T were actually increasing in *M. sexta* tissues, we examined levels in midgut, fat body, and carcass of larvae fed on control diets and on diets supplemented with 0.1% antioxidant (Table 1). In the case of both β-carotene and vitamin E, the increased dietary antioxidant level resulted in a corresponding increase in tissue levels. In the standard *M. sexta* diet the level of β-carotene and vitamin E is extremely low (<0.0001%), and this is close to the levels observed in the tissues of control groups. For vitamin E, increasing the dietary concentration by 1000-fold resulted in 445- to 2644-fold increases in tissue levels, while increases in dietary β-carotene resulted in tissue β-carotene levels that were only 48- to 57-fold higher.

DISCUSSION

We have demonstrated that the lipid-soluble antioxidants β-carotene and vitamin E, when administered in artificial diets, substantially reduce the mortality associated with treatment of *M. sexta* larvae with the phototoxin α-T. Ascorbic acid provided no additional protection when dietary levels were

TABLE 1. TISSUE LEVELS OF ANTIOXIDANTS IN *M. sexta* LARVAE

	Vitamin E[a]			β-Carotene[a]		
	Midgut	Fat body	Carcass	Midgut	Fat body	Carcass
Control[b]	3.3 ± 0.4	7.0 ± 1.4	2.0 ± 0.2	0.2 ± 0.1	N.D.	0.3 ± 0.1
0.1% antioxidant in diet	4980 ± 1259	3103 ± 97	5209 ± 60	5.7 ± 1.4	1.0 ± 0.3	18.9 ± 3.2
-Fold increase	1491	445	2644	48		57

[a] μg/g fresh weight tissue ($X \pm$ SEM, $N = 3$ samples). N.D. not detected.
[b] Fresh diet was analyzed and found to contain approximately 1 μg/g vitamin E and 0.1–0.5 μg/g β-carotene.

increased, whereas a reduction in the control levels resulted in much higher mortality. This latter result may be due, however, to a decreased fitness of the larvae. Ascorbic acid is an essential nutrient for most herbivorous insects (Dadd, 1985), and in *M. sexta* suboptimal dietary ascorbic acid has been shown to cause abnormal growth and development (Kramer et al., 1978). Ascorbic acid is capable of scavenging activated oxygen species such as superoxide anions, although its efficiency is orders of magnitude lower than superoxide dismutase (Levine and Kidd, 1986). Ascorbic acid also can react with and reduce the tocopherol quinone radical resulting from the oxidation of tocopherol and in this way regenerate reduced tocopherol, which is an interceptor of free radicals and singlet oxygen (Witting, 1976). The fact that increased dietary ascorbic acid provided no additional protection against α-T might be related to its hydrophilic character [in contrast to the lipophilicity of α-T (log $P = 5.7$)]. In the housefly *Musca domestica*, ascorbate actually enhanced slightly the phototoxic effect of erythrosine B treatment of larvae (Robinson and Beatson, 1985).

Unlike ascorbic acid, vitamin E appears to be an essential dietary requirement in far fewer species (Dadd, 1985). Vitamin E has been shown to be required for normal adult reproductive function in *Spodoptera exempta* (David and Ellaby, 1975) and in crickets (McFarlane, 1972). An important role for vitamin E in insects is likely as a general antioxidant since it is an excellent scavenger of activated oxygen species (Foote et al., 1978). It also may have a role in the conservation of tissue polyunsaturated fatty acids, as has been suggested for *Pieris brassicae* (Turunen, 1976). In our study, vitamin E was found to be an extremely effective antioxidant capable of protecting *M. sexta* larvae against lethal oxidative damage from α-T. The toxicity of α-T to insects is believed to be due primarily to its strong production of singlet oxygen and subsequent damage to essential membranes (Bakker et al., 1979; Arnason et al., 1981a, b; Scaiano et al., 1989). McRae et al. (1985) have shown that antioxidants can markedly reduce α-T-induced lipid peroxidation in egg lecithin liposomes. In the case of *M. sexta*, treatment of larvae with sublethal levels of α-T has been shown to cause black lesions of the cuticle (Downum, 1986; Champagne et al., 1986) and to cause rapid destruction of midgut epithelial cells (Sen et al., 1990). The lipid-soluble nature of vitamin E and the ability of *M. sexta* tissues to rapidly accumulate large amounts of this antioxidant from their diet (Table 1) may account for its protective effect.

Carotenoid pigments also can function as effective quenchers of singlet oxygen and other oxygen-centered radicals (Foote and Denny, 1968; Krinsky and Deneke, 1982). β-Carotene has the highest quenching rate known for any quencher of singlet oxygen ($2–3 \times 10^{10}$ M sec) (Foote, 1987). Carotenoids may react directly with peroxy and alkoxy radicals and hence prevent lipid peroxidation (Halliwell and Gutteridge, 1985). β-Carotene has been shown to protect against phytotoxicity induced by 8-MOP in mammals (Giles et al.,

1984). In insects, carotenoids have been examined from a variety of tissues (see, for example, Feltwell, 1978; Goodwin, 1980), and the dominant carotenoids found are lutein and β-carotene (Feltwell and Rothschild, 1974). Large variations exist in the ability of some insects to absorb these carotenoids (Kayser, 1985), variations that also are found in insects reared on identical host plants (Feltwell and Rothschild, 1974). In terms of their function in insects it is known that deprivation of either vitamin A or suitable carotene precursors results in an impairment of the visual and light responses in several species (Veerman, 1980). In addition to their role in insect warning coloration, it has been suggested that they also may have a photoprotective role (Feltwell and Rothschild, 1974). In a short report, Poff (1976) observed that cryptic *Leptinotarsa* species had low concentrations of carotenoids and a lower carotenoid-to-xanthophyll ratio than in their host plants and conversely that aposematic species had higher concentrations of carotenoids and a higher ratio of carotenoids to xanthophylls. Poff (1976) suggested this high level of carotenoids might have a protective role against incoming radiation.

In our study, β-carotene effectively protected *M. sexta* at a dietary concentration of 0.1%, a concentration frequently found in plants in nature (Bureau and Bushway, 1986). It is not known whether *M. sexta* might be able to attain this level of β-carotene and/or protection by feeding on its typical host plant, tobacco. Nevertheless, we have demonstrated that dietary antioxidants such as β-carotene can dramatically alter the sensitivity of this insect herbivore to a plant phototoxin.

Our results with β-carotene and vitamin E are consistent with the view that the lethal damage caused by α-T is due primarily to $^1O_2$ formation, as both antioxidants are excellent quenchers of this species of O_2. However, type I (electron transfer) and type II (energy transfer) processes are always in competition (Foote, 1987), hence we cannot rule out that the phototoxicity of α-T is due in part to a free-radical mechanism. Vitamin E is also a good radical scavenger. α-T is capable of O_2^- production (Kagan et al., 1989), although laser flash photolysis studies (Scaiano et al., 1989) indicate that electron transfer accounts for only 1% of the activated O_2. Perhaps specific scavengers and quenchers of different activated species of O_2 could be used to resolve this question.

We also have been investigating antioxidant defenses in phototoxin-tolerant insects. Larvae of the chrysomelid beetle *Chrysolina hyperici*, for example, are specialist feeders on *H. perforatum*, which contains hypericin, a strong generator of singlet oxygen (Knox and Dodge, 1985). These larvae, although primarily using light-avoidance as a defense mechanism against phototoxicity (see Fields et al., 1990), have been observed to have a brightly colored (orange-red) fat body. HPLC analysis of last-instar larvae (results not shown) indicated levels of β-carotene of 336.2 ± 45.7 μg/g ($X \pm$ SEM, $N = 3$), levels that are

two- to fivefold higher than have been observed in many coleopteran and lepidopteran larvae (Feltwell and Rothschild, 1974). The protection provided by β-carotene to *M. sexta* suggests that high levels of β-carotene in *C. hyperici* also may protect these insects from host-plant phototoxins.

A number of factors may be involved in explaining the extreme sensitivity of *M. sexta* larvae to phototoxins, not the least of which might be the low level of lipid-soluble antioxidants in its artificial diet. *M. sexta* also has been shown recently to have a relatively low complement of the antioxidant enzymes superoxide dismutase, catalase, and glutathione reductase (Aucoin et al., in preparation), and this may contribute to its sensitivity. Physical factors such as the amount of light penetration through the cuticle also may play an important role. Berenbaum (1987) has observed that a black mutant strain of this species is less sensitive to phototoxins than normally pigmented larvae. As well, *M. sexta* is an oligophagous herbivore and may not have had sufficient exposure to phototoxins in its natural diet breadth to develop an adaptive response in the form of specific defenses.

The fact that *M. sexta* can be protected from the toxicity of α-T suggests that the sensitivity of other insect herbivores to phototoxins may be dependent in part on the nature and level of plant chemicals in their diet that can act as antioxidants. This would depend also on the ability of insects to absorb and utilize these chemicals. Antioxidants may have played an important role in the ability of some insects to develop successfully on phototoxic plants.

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VARIATION IN TANNIN ACTIVITY OF ACORNS OF SEVEN SPECIES OF CENTRAL FLORIDA OAKS

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Abstract—Acorns of seven sympatric species of oaks (*Quercus* spp.) occurring in central Florida were compared for protein-precipitating ability (PPA). Husks and cotyledons of green and ripe acorns were analyzed. Few significant differences were found between green and ripe acorns of the same species. Cotyledons were significantly higher than husks in PPA in most of the species compared. All species exhibited high intraspecific variability in PPA. Significant differences between species were found in green and ripe cotyledons and in green husks. With the exception of *Q. minima*, PPA of ripe cotyledons was higher in the subgenus *Erythrobalanus* (red oaks) than the subgenus *Quercus* (white oaks).

Key Words—Acorns, defensive compounds, Fagaceae, oaks, phenolics, *Quercus*, seasonality, tannins, variability.

INTRODUCTION

The widely cited deleterious effects of tannins on herbivores can be placed into three categories: (1) The ability of tannins to precipitate proteins and other cellular components in vitro (Swain, 1979; Hagerman and Klucher, 1986) may indicate that digestibility of plant components is affected (Feeny, 1970; Short, 1976; but see Bernays, 1981). (2) Precipitation of digestive enzymes by tannins can reduce the enzymes' effectiveness (Goldstein and Swain, 1965; Feeny,

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1969). (3) Tannins may have direct toxic effects by attacking the gut epithelium (Singleton and Kratzer, 1973; Bernays, 1981) and causing death by liver necrosis when consumed in high concentrations (Singleton and Kratzer, 1973). The astringent qualities of tannins (Bate-Smith, 1972) also may be responsible for feeding deterrence in animals, e.g., giant tortoises (Swain, 1978a), deer (Cooper-Driver et al., 1977), *Colobus* monkeys (Swain, 1978a), gorillas (Harborne, 1982), and moth larvae (Feeny, 1970). Extensive reviews of the various effects of tannins on herbivores can be found in Levin (1976), Swain (1977, 1978a, 1979), Bernays (1981), and Harborne (1982). Tannins may function to reduce fungal and microbial attacks on plant tissue as well (Swain, 1978b).

Acorns are an important food source to many animals throughout North America (Martin et al., 1951). However, the reportedly high tannin concentrations of some acorns (Ofcarcik and Burns, 1971) may affect their use by animals (Martin et al. 1951); this has been suggested as a partial explanation of the acorn-storing behaviors of gray squirrels (Smallwood and Peters, 1986).

Tannin concentration can vary widely between individuals of a species as a result of genetic and environmental differences and can vary through time on a single tree because of seasonal and age-related change or damage-induced tannin production (Feeny and Bostock, 1968; Schultz et al., 1982; Baldwin et al., 1987). Accurately assessing tannin effects on the use by animals of different species' acorns is difficult, as tannin concentration has been measured in only a few oak species. Little information is available on tannin concentration and protein-precipitating ability (PPA) of green acorns as compared to ripe acorns, and few data are available on acorn husk astringency or tannin concentration. Some seed husks are known to have high tannin concentrations (Swain, 1979). Acorn husk astringency could be an important factor in acorn use to consumers, such as rodents, bears, deer, and hogs, that gnaw through the husk or consume the entire acorn to reach the cotyledon. Based on the relatively small number of tannin concentration analyses available (Ofcarcik and Burns, 1971; Short, 1976), acorns fall into two categories coinciding with the two subgenera of oaks. *Erythrobalanus* (black or red oak) acorns characteristically have higher tannin concentrations and *Quercus* (white oak) acorns have lower tannin concentrations (Ofcarcik and Burns, 1971; Elias, 1971).

This paper reports PPA analysis results for acorns of major oak species occurring in natural vegetative associations in south central Florida. These species' nutrient compositions have been reported (Abrahamson and Abrahamson, 1989), but tannin effects have not. Oaks are dominant canopy or shrub layer members of these communities, and their acorns provide an important food source for many vertebrates in the region, including wild turkeys (*Meleagris gallopavo*), scrub jays (*Aphelocoma coerulescens*), blue jays (*Cyanocitta cristata*), red-bellied woodpeckers (*Melanerpes carolinus*), black bears (*Ursus*

americanus), rodents such as gray squirrels (*Sciurus carolinensis*), southern flying squirrels (*Glaucomys volans*), Florida mice (*Podomys floridanus*), mice of the genus *Peromyscus*, white-tailed deer (*Odocoileus virginianus*), and invertebrates, such as curculionid larvae. Six oak species (Chapman's oak, *Quercus chapmanii*; sand live oak, *Q. geminata*; scrub oak, *Q. inopina*; turkey oak, *Q. laevis*; runner oak, *Q. minima*; and myrtle oak, *Q. myrtifolia*) found at Archbold Biological Station occur in southern ridge sandhills, pine flatwoods, sand pine scrub, and scrubby flatwoods communities, as described by Abrahamson et al. (1984). Laurel oak (*Quercus laurifolia*) has been planted in a few sites on the study area and occurs naturally in mesic forest communities nearby. *Quercus chapmanii*, *Q. geminata*, and *Q. minima* are members of the *Quercus* subgenus; *Q. inopina*, *Q. laevis*, *Q. laurifolia*, and *Q. myrtifolia* are members of the *Erythrobalanus* subgenus (Elias, 1971; Wunderlin, 1982).

METHODS AND MATERIALS

We collected green and ripe acorns from trees during October and November 1986. Husks and cotyledons of individual acorns were used as assay samples. Multiple acorns were not collected from a single tree. Each sample had three replicates. Sample sizes of 16 to 20 undamaged acorns were used for each species, except *Q. minima*, in which eight acorns were used.

The assay method was essentially that of Hagerman (1987). It is a radial diffusion assay in which a tannin-bearing extract is introduced into an agarose gel containing a protein; the tannin in the extract precipitates the protein, forming a visible white ring around the introduction site. The square of the diameter of the precipitation ring is linearly correlated with the extract's PPA (Hagerman, 1987). The method was used because it is relatively simple and well suited to processing many samples. It also has the benefit of directly measuring the degree of protein precipitation caused by a sample. This may be biologically more relevant than indicating the actual quantity of tannins present (Wisdom et al., 1987). Because different types of tannins have different effects on proteins (Swain, 1978b, 1979; Hagerman and Klucher, 1986), simply quantifying the amount of tannins may give no idea of their effects on herbivores.

We prepared acorn extracts by separating the acorns' husks from cotyledons, chopping cotyledons into 2 to 4-mm cubes, and drying cotyledons and husks to constant weight at 45–50°C. We ground dried materials in a Wiley mill to pass through a 20-mesh screen. Approximately 0.1 g of ground material, weighed to the nearest 0.1 mg, was used in each sample.

Heat drying oxidizes tannins and binds them to cell membranes, reducing their protein-binding activity (Swain, 1979). In 1989, we determined the approximate reduction of activity in cotyledons of five species (*Q. chapmanii*,

TABLE 1. MEANS AND STANDARD DEVIATIONS OF MASS CHANGE AND WET AND DRY ACORN PROTEIN PRECIPITATION ABILITY (PPA) VALUES[a] IN FIVE SPECIES[b] OF OAKS

Species	Ratio of dry to wet acorn mass		Mean acorn PPA				Predicted PPA[c]	Ratio of predicted to dry acorn PPA means
			Wet		Dry			
	Mean	SD	Mean	SD	Mean	SD		
Q. chapmanii	0.503	0.11	2.032	0.48	2.850	0.55	4.043	1.419[d]
Q. laevis	0.546	0.07	1.484	0.23	1.940	0.49	2.718	1.401[d]
Q. geminata	0.766	0.11	2.172	0.60	2.640	0.70	2.836	1.074
Q. inopina	0.918	0.01	1.646	0.16	1.664	0.42	1.792	1.077
Q. myrtifolia	0.942	0.01	1.462	0.28	1.654	0.40	1.551	0.938

[a] mm^2 protein precipitated/mg dry weight.
[b] N for all species = 10.
[c] Calculated as (mean wet PPA)/(ratio of dry to wet acorn mass).
[d] $P < 0.05$ (t test).

Q. geminata, *Q. inopina*, *Q. laevis*, and *Q. myrtifolia*; Table 1) collected in September and October 1989. Ten acorns of each species were hulled and split into cotyledons. One cotyledon of each acorn was oven-dried to constant weight at 45–50°C (approximately 48 hr). The other cotyledon was refrigerated in a sealed vial for the duration. All cotyledons were ground in a chilled mortar and pestle and refrigerated until extraction.

All ground materials were extracted with 50% aqueous methanol for 2 hr at room temperature (approximately 23°C) using 0.5 ml solvent per sample (Hagerman, 1987). Samples extracted for longer periods, using 1.0 ml solvent, did not show significantly greater PPA. Details of the remaining procedure are in Hagerman (1987).

RESULTS

Changes in dried and undried ("wet") cotyledon PPA were correlated with cotyledon water content, measured as dry weight proportion of wet weight ($r^2 = 0.977$, $P < 0.01$). For three species, dry cotyledon PPA could be predicted using wet PPA values by dividing wet PPA by weight retained by dry cotyledons (Table 1). The only species showing unpredicted reduction in PPA between dried and undried cotyledons were *Q. chapmanii* and *Q. laevis*, which also had the highest water contents of species tested (Table 1). Because these species had among the highest PPA values, we feel that sample drying minimized interspecific differences between assayed acorns, leading to more conservative results. Acorn cotyledons analyzed in 1989 showed patterns very similar to the

1986 acorns; only in *Q. geminata* were significant differences in PPA between the two years found ($P < 0.05$, one-way ANOVA). We were unable to test *Q. minima* or *Q. laurifolia* acorns. Assuming their responses fall anywhere within the range of tested acorns, no rankings of acorn components changed because of drying.

Kruskal-Wallis tests (Sokal and Rohlf, 1981) of dried acorn components (green and ripe husks; green and ripe cotyledons) showed significant differences ($P < 0.05$) between species for ripe husks and for both green and ripe cotyledons (Table 2). In all species, acorn cotyledons had a higher mean PPA than acorn husks, regardless of ripeness. PPA varied considerably between individuals within species, coefficients of variation ranging from 16.7% (*Quercus inopina* green husk, *Q. chapmanii* ripe husk) to 109.9% (*Q. laevis* green husk). We used a nonparametric multiple range test (Dunn, 1964) to discover where significant differences existed between the component means. Because of the relatively large number of comparisons necessary when testing for differences among seven means, the individual comparison significance level was set at $P < 0.0012$ ($0.05/2 \times$ number of comparisons) to achieve an overall 0.05 significance level. The analysis indicated that *Q. chapmanii* husks were significantly lower in PPA than *Q. minima*, *Q. laurifolia*, *Q. inopina*, or *Q. myrtifolia* husks. Among green acorn cotyledons, *Q. inopina* and *q. myrtifolia* were significantly lower in PPA than *Q. laurifolia*, while among ripe acorn cotyledons *Q. chapmanii*, *Q. geminata*, *Q. myrtifolia*, and *Q. inopina* were significantly lower in PPA than *Q. laurifolia*.

Green cotyledons had significantly higher PPA than green husks in *Q. chapmanii*, *Q. geminata*, *Q. myrtifolia*, and *Q. laurifolia*. Ripe cotyledons had significantly higher PPA than ripe husks in all species except *Q. minima*. Green

TABLE 2. MEANS AND STANDARD DEVIATIONS OF PROTEIN PRECIPITATION ABILITY (PPA) VALUES[a] OF DRIED GREEN AND RIPE HUSKS AND GREEN AND RIPE COTYLEDONS OF ACORNS OF SEVEN OAK SPECIES

Species	Husk			Cotyledon		
	Green	Ripe	% change	Green	Ripe	% change
Q. chapmanii	0.918 (0.472)	0.318 (0.053)	−65.4	3.254 (1.605)	1.209 (0.346)	−62.8
Q. geminata	1.114 (0.254)	0.598 (0.206)	−46.3	1.823 (0.846)	1.312 (0.365)	−28.0
Q. minima	1.043 (0.254)	1.120 (0.766)	+7.4	3.498 (1.314)	2.401 (1.360)	−31.4
Q. inopina	1.000 (0.167)	0.818 (0.340)	−18.2	1.371 (0.370)	1.730 (0.873)	+26.2
Q. laevis	1.328 (1.460)	0.515 (0.298)	−61.2	2.823 (1.317)	2.201 (0.473)	−21.4
Q. laurifolia	0.740 (0.300)	0.773 (0.135)	+4.4	4.304 (1.281)	6.077 (1.261)	+41.2
Q. myrtifolia	0.833 (0.224)	0.745 (0.352)	−10.6	1.387 (0.288)	1.436 (0.214)	+3.5

[a] mm^2 protein precipitated/mg dry weight.

husks were significantly lower in PPA than ripe husks in *Q. chapmanii* and *Q. geminata*. Only in *Q. chapmanii* were green cotyledons significantly higher in PPA than ripe cotyledons.

DISCUSSION

"Tannins" refers to a diverse group of phenolic compounds found in all classes of vascular plants. On the basis of chemical structure, tannins can be divided into two major groups, hydrolyzable tannins and condensed tannins. Hydrolyzable tannins are derivatives of gallic acid and simple sugars and are relatively uncommon in plants. Condensed tannins are flavonoids and flavonoid polymers, compounds that also serve as pigments in many plants and are widespread (Swain, 1979). The ecologically important feature of tannins with which we are principally concerned is their protein-precipitating ability (PPA). It is this quality that is thought to be responsible for herbivore deterrence. This ability is not constant within or between classes of tannins (Bate-Smith, 1977). Therefore measures of tannin concentration, rather than protein precipitating ability, may be misleading or irrelevant if they are used as indicators of palatability or nutritional quality (Martin and Martin, 1982; Wisdom et al., 1987). The assay used in these experiments is an in vitro measure of tannin activity, measured as PPA. It does not distinguish between hydrolyzable and condensed tannins, so it is not possible using this method to state the exact composition of tannins in the tested species. However, it does directly measure relative PPA. Because most previous tannin research does not report results in terms of PPA, direct comparison of our results with those of others may be difficult.

The lack of research on acorn chemistry also makes comparison with other studies difficult. Previous acorn studies (Wainio and Forbes, 1941; Ofcarcik and Burns, 1971; Koenig and Mumme, 1987; Koenig and Heck, 1988) did not assess changes in tannins through time. Only Koenig and Heck (1988) measured amounts of hydrolyzable and condensed tannins in acorns. Thus comparisons are only possible with studies of seasonal tannin change in other plant parts, particularly foliage.

The assay results show a high degree of within-species variation in PPA, a pattern that is similar to previous tannin assays. Ofcarcik and Burns (1971) and Sork et al. (1983) found marked variation in acorn tannin levels between individual trees within species. Similar variation has been found in tannin levels in birch and maple foliage (Schultz et al., 1982; Baldwin et al., 1987). Possible sources of such variation were summarized by Baldwin et al. (1987) and include: (1) responses of individual tree or parts of trees to damage; (2) seasonal change; (3) plant component age; (4) genotypic differences; and (5) site variation. Tannin concentration may increase in plant components subjected to herbivory or

other forms of damage (Baldwin and Schultz, 1983). A response by the oak to damage is possible in this case, because other studies have shown damage-induced increases in tannin concentration in the undamaged foliage of damaged trees (Schultz and Baldwin, 1982). All trees we examined had acorns infested with *Curculio* larvae.

Some of the observed variation may have an ontological basis. Attempts were made to collect acorns as similar in size and husk color as possible, but because the exact date of seed set was not known, the acorns used ranged in maturity. Variation in tannin production within oak populations may be heritable, as it is known to be in sorghum (Woodruff et al., 1982), and it may also be influenced by small-scale site variation. Baldwin et al. (1987) found birch and maple tannin variation was as large within small geographical areas as in distantly separated parts of the species' ranges. Environmental variation over a small geographic range can affect plant fruit chemistry (Levey, 1987). A number of soil and moisture regimes are known in the area where samples were collected (Abrahamson et al., 1984) and conceivably could affect acorn PPA.

Although little significant change was found in the PPA of species during ripening, green cotyledon PPA patterns were very different from ripe cotyledon PPA. Faeth (1986) reported a complex pattern in the quantities of hydrolyzable and condensed tannin in *Quercus robur* foliage. Hydrolyzable tannins dropped sharply in spring, remaining afterwards at a fairly constant level, while condensed tannins rose steadily until late summer, when they also remained nearly constant at a lower concentration than hydrolyzable tannins. There is no reason to expect the distribution and patterns of PPA to be identical between foliage and fruits, but this pattern would probably have the effect of decreasing or holding constant PPA through time, a result similar to ours.

Acorn husks proved to be relatively low in PPA as compared to acorn cotyledons, although some species were highly variable in this regard. High tannin concentration has been reported in the seed coats of some plants (Swain, 1977). Low husk astringency as compared to cotyledon astringency is one pattern that would be expected if PPA functions to protect valuable plant components from fungal and bacterial attack; in this case the ability of husks to ward off fungal attack may be unimportant. The acorn husks tested fit this pattern. It seems unlikely that these acorns' low husk astringency could function effectively as a deterrent to rodents and other animals that gnaw or pierce the husk to reach the cotyledon.

Ofcarcik and Burns (1971) found a strong correlation between tannin content and taxonomy; *Quercus* subgenus oaks were all relatively low in tannin content (less than 2.1% dry weight) while *Erythrobalanus* acorns were all relatively high (5.7% dry weight or greater). Short (1976) presented circumstantial evidence based on feeding preferences of gray squirrels that *Quercus* acorns as a group have a lower PPA than *Erythrobalanus* acorns. However, the species

of the two subgenera in this study do not always clearly fall into two groups on the basis of PPA. Our PPA results for ripe cotyledons are consistent with those of Ofcarcik and Burns (1971), except for *Q. minima*. The variation in PPA in *Q. minima* samples and the small sample size make it difficult to reach a conclusion on the status of this species' PPA. Conversely, among the green acorns, the species with greatest PPA (*Q. laurifolia*) as well as the two species lowest in PPA (*Q. inopina* and *Q. myrtifolia*) are *Erythrobalanus* acorns, whereas *Q. minima* and *Q. chapmanii*, second and third highest in PPA, are both subgenus *Quercus*. The commonly observed pattern of high-tannin *Erythrobalanus* acorns and low-tannin *Quercus* acorns may hold true only in fully ripe acorns.

Koenig and Mumme (1987) compared soluble tannin in four western oak species, two in *Quercus* (*Q. lobata*, *Q. douglasii*) and two in *Erythrobalanus* (*Q. agrifolia*, *Q. kelloggii*). Although they found little variation among species (all values between 0.37% and 0.46% dry weight), *Erythrobalanus* acorns occupied the upper end of this range. Koenig and Heck (1988) found much greater amounts of hydrolyzable tannins in *Q. agrifolia* than in *Q. chrysolepis* (an "intermediate" oak, subgenus *Protobalanus*) or *Q. lobata*. Some of the disagreement between studies probably results from differences in method. However, our results indicate that patterns of PPA in ripe acorns may not give a reliable picture of acorn PPA during development.

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GENETIC ASPECTS OF INTERPOPULATIONAL DIFFERENCES IN PHEROMONE BLEND OF CABBAGE LOOPER MOTH, *Trichoplusia ni*

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Abstract—The genetic basis of interpopulational differences in the pheromone blend emitted by the cabbage looper moth, *Trichoplusia ni* (Hübner), was examined by crossing individuals from a field-derived population (P_1) with individuals from a long-maintained laboratory colony (P_2). These colonies differed in the emission rate and relative proportions of four of the five known minor pheromone components, but not in the emission rate of the major component, (Z)-7-dodecenyl acetate (Z7–12 : Ac). These differences in pheromone blend were quantitatively small but biologically significant, because in the field, males responded preferentially to traps baited with a pheromone blend that is similar to that emitted by P_1 females relative to a blend similar to that emitted by P_2 females. In initial crosses, variation in the quantity and quality of pheromone blends among families of P_1, P_2, and F_1 hybrid females was examined. In F_1 females the relative proportions (quantity relative to the major component) of (Z)-5-dodecenyl acetate (Z5–12 : Ac) and (Z)-7-tetradecenyl acetate (Z7-14 : Ac) were intermediate to parental lines. In a second more extensive set of crosses, analyses included P_1, P_2, F_1, F_2, and selected backcrosses. The relative proportion of Z5–12 : Ac, Z7–14 : Ac, and Z9–14 : Ac emitted by F_1 females were intermediate to parental lines. The frequency distributions of relative proportions of these components emitted by females were not consistent with those expected under a single autosomal or sex-linked gene hypothesis, suggesting that more than one gene is involved in the quantitative differences in the pheromone blend.

Key Words—Genetics, sex pheromone, Lepidoptera, Noctuidae, *Trichoplusia ni*, cabbage looper moth, reproductive isolation.

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INTRODUCTION

Intra- and interpopulational variation in chemical communication systems have been documented in several species of insects (Lanier et al., 1972; Klun and cooperators, 1975; Klun and Maini, 1979; Miller and Roelofs, 1980; Collins and Cardé, 1985; Löfstedt et al., 1986; Miller et al., 1989). In Lepidoptera, variation in the pheromone blend within a population is usually small, suggesting that stabilizing selection between signaler and responder may be important (Cardé, 1986; Roelofs et al., 1986). Documented examples of geographical variation between populations may be explained by genetic drift and/or directional selection. In a few species, the genetic basis for these differences has been examined, either through crossing experiments (Grula and Taylor, 1979; Klun and Maini, 1979; Roelofs et al., 1987) or selection experiments (Roelofs et al., 1986; Collins and Cardé, 1989).

The chemical communication system of the cabbage looper moth, *Trichoplusia ni* (Lepidoptera: Noctuidae), should be a good model system for studying the genetic basis for control of pheromone blend and how changes in pheromone blend might be involved in speciation or the potential for evolution of resistance to synthetic pheromone (Cardé, 1976). The sex pheromone of the cabbage looper moth was identified as (Z)-7-dodecenyl acetate (Z7–12 : Ac) (Berger, 1966) but is now known to consist of this major component and dodecyl acetate (12 : Ac), (Z)-5-dodecenyl acetate (Z5–12 : Ac), 11-dodecenyl acetate (11–12 : Ac), (Z)-7-tetradecenyl acetate (Z7–14 : Ac), and (Z)-9-tetradecenyl acetate (Z9–14 : Ac) (Bjostad et al., 1984; Linn et al., 1984). We recently reported that females from a long-maintained laboratory colony emitted significantly higher proportions of several minor components (Haynes and Hunt, 1990a) than females from three field populations. These significant differences in the pheromone blend ratios were relatively subtle when compared with the dramatic change in pheromone blend emitted by mutant females discovered within the same laboratory colony (Haynes and Hunt, 1990b). Previously, we determined by crossing experiments that the change in the pheromone blend in the mutant females was controlled by a single autosomal gene. In this report we provide evidence that the subtler differences between our laboratory and field colonies are probably controlled by more than one gene. In addition, we show that these subtle differences are biologically significant in terms of attraction of male moths under field conditions.

METHODS AND MATERIALS

Insect Colonies. The "field" colony (P_1) originated from female *T. ni* collected in Riverside, California (Haynes and Hunt, 1990a). This colony was maintained for ca. 10 generations following the same protocol as that used for

the "laboratory" (P_2) colony, which has been maintained using the procedures of Shorey and Hale (1965). The P_2 colony also originated from field-collected adults near Riverside but has been reared for more than 100 generations without the introduction of any individuals from field populations. Pupae were separated according to sex and placed in 3.8-liter paper cartons. Emerged adults were removed daily and transferred to additional cartons. Pupae and adults were housed in environmental chambers that were maintained at 27°C during the 16-hr photophase and 21°C during the 8-hr scotophase. Voucher specimens of P_1 and P_2 *T. ni* have been deposited in a reference collection housed in the Department of Entomology, University of Kentucky.

Field Tests. To determine if previously documented differences in the emitted blend ratios of the two colonies were biologically significant, rubber septa were impregnated with synthetic blends formulated to release pheromone components at a rate similar to either P_1 or P_2 females. For the P_1 blend a dose of 500 μg of the major component, Z7–12:Ac, and 25, 50, 15, 15, 10 μg of 12:Ac, Z5–12:Ac, 11–12:Ac, Z7–14:Ac, and Z9–14:Ac, respectively, in 50μl of CS_2 resulted in emission rates similar to mean values reported by Haynes and Hunt (1990a) for this population. The P_2 blend consisted of the same amount of the major component and 35, 75, 15, 40, and 25 μg of the five minor components listed above, respectively. During October 1988, Pherocon 1C traps were baited with one septum containing either the P_1 blend, the P_2 blend, or a blank septum (control). Traps were placed at least 20 m apart in a 3.0-ha alfalfa field at the University of Kentucky's Spindletop farm, Fayette County, Kentucky. The openings of the traps were approximately 10–20 cm above the plant canopy. Traps were deployed in a randomized block design (20 blocks over four nights), and the number of moths captured was determined daily. Traps were refitted with new sticky trap bottoms, and their positions were rerandomized each night.

Crosses. An initial set of crosses was done to quantify the variability of each pheromone component emitted by females among families of the P_1 and P_2 parental lines and F_1 hybrids. In a second set of crosses, pheromone phenotypes of females from families of P_1, P_2, F_1, F_2, and F_1 to P_1 (BC_1) and F_1 to P_2 (BC_2) backcrosses were determined. Single pair matings were accomplished by housing one male and one female moth in 473-ml cartons lined with blotter paper for egg laying and closed with nylon screened lids. Rearing procedures followed those described previously. The types of crosses and number of families and individuals examined for both sets of crosses are shown in Table 1. Just prior to the initiation of the scotophase, 3- to 4-day-old adult females were placed individually in hardware cloth cages (4 cm diameter × 4 cm height × 3 mm mesh) closed with aluminum lids in preparation for pheromone analysis.

Volatile Analysis. Collection of volatiles from females followed procedures described by Haynes and Hunt (1990a), who used a method modified from Baker et al. (1981). During the second through eighth hour of the scoto-

TABLE 1. CROSSES, NUMBER OF FAMILIES, AND TOTAL NUMBER OF CABBAGE LOOPER FEMALES FOR WHICH PHEROMONE BLENDS WERE QUANTIFIED IN TWO EXPERIMENTS

Cross type[a]	Parentage[b] Female × Male	Number of families examined	Total number of females examined
Exp 1			
P_1	$P_1 \times P_1$	8	44
P_2	$P_2 \times P_2$	7	33
F_{1a}	$P_1 \times P_2$	3	19
F_{1b}	$P_2 \times P_1$	9	50
Exp. 2			
P_1	$P_1 \times P_1$	1	34
P_2	$P_2 \times P_2$	1	36
F_{1a}	$P_1 \times P_2$	1	33
F_{1b}	$P_2 \times P_1$	1	31
F_{2a}	$P_1P_2 \times P_1P_2$	1	30
F_{2b}	$P_2P_1 \times P_2P_1$	1	36
F_{2c}	$P_1P_2 \times P_2P_1$	1	28
F_{2d}	$P_2P_1 \times P_1P_2$	1	37
BC_{1a}	$P_1P_2 \times P_1$	1	33
BC_{1b}	$P_2P_1 \times P_1$	1	32
BC_{2a}	$P_1P_2 \times P_2$	1	18
BC_{2b}	$P_2P_1 \times P_2$	1	35

[a] P_1 and P_2 refer to individuals from the field-derived population and the laboratory colony, respectively. BC_1 and BC_2 refer to backcrosses of F_1 females to P_1 or P_2 males, respectively.
[b] Where two letters are used to indicate the type of parent used in a cross, the first letter represents the female and second letter represents the male parentage.

phase, a period during which calling behavior was observed under the experimental conditions (Hunt and Haynes, unpublished results), a female was removed from an environmental chamber and inserted abdomen first into a glass holder. The glass holder and all-glass collector were described by Haynes and Hunt (1990a) and Baker et al. (1981). A stream of nitrogen gas flowed through the collector at a rate of 120 ml/min. After a 10-min collection period, 10 ng of an internal standard, (*E*, *Z*)-4, 7-tridecadienyl acetate, was added to the adsorbent glass wool surface at the distal end of the collector, and then the collector was rinsed out with two 50-μl aliquots of CS_2. This volume was reduced to ca. 1 μl before it was injected into a 30-m Carbowax 20 M column housed in a Hewlett-Packard 5890A GC linked to a Hewlett-Packard Mass Selective Detector. Details on GC programming, MS tuning, and the establishment of standard curves are given by Haynes and Hunt (1990a). The lower analytical limit of this technique was ca. 0.01 ng.

RESULTS AND DISCUSSION

The differences between parental strains in the emitted blend ratios of the minor pheromone components were biologically significant. In the field, traps ($N = 20$) baited with rubber septa that released a blend of synthetic pheromone components similar to P_1 females captured 4.4 $\pm$ 0.7 (SE) male *T. ni* per trap per night. Traps ($N = 20$) baited with septa emitting a blend similar to P_2 females captured 2.8 $\pm$ 0.6 (SE) males per trap per night. Control traps ($N = 20$) captured no male *T. ni*. Thus the blend resembling that emitted by females from field populations captured significantly more male *T. ni* (Sidak's multiple comparison test, $P < 0.05$, preceded by two-way ANOVA, $F = 24.44$, $df = 2,38$, $P < 0.0001$). Under field conditions, females that emit a blend similar to the optimum blend for the males' response would have a selective advantage. Conversely, males that respond preferentially to the blend released by the average female would have a reproductive advantage. Under laboratory conditions, this type of normalizing selection may be relaxed, allowing the genetic divergence that has resulted in the differences between the two populations.

Females from the two parental colonies did not differ in the emission rate of Z7-12 : Ac, the major component (Figure 1). Analyses of minor components based on their proportion relative to the major component (quantity of a minor component divided by the quantity of the major component, Z7-12 : Ac) show that all except 11-12 : Ac were higher in P_2 females than in P_1 females (Figure 1). These differences were consistent with earlier results obtained in a study of interpopulational variation in this species in that the emitted blend ratios of pheromone components from females from the P_1 colony are similar to those of females from field populations from Kentucky and Florida (Haynes and Hunt, 1990a). Since the P_2 colony has been maintained in laboratory culture much longer (>10 years) than the P_1 colony, it appears most likely that the pheromone blend ratios of P_2 colony have diverged from the typical pattern seen in field populations. The differences between the two parental populations could have arisen through selection or genetic drift.

An analysis of volatiles emitted by females from crosses between the two parental lines has given us information on the inheritance of the pheromone blend ratios. There was no significant difference in the relative proportion of 11-12 : Ac in the parental lines, and as expected F_1 hybrids were similar to the parental lines in this characteristic. In contrast, the relative proportions of Z5-12 : Ac, Z7-14 : Ac, and Z9-14 : Ac emitted by F_1 females were intermediate to that found in P_1 and P_2 parental lines (Figure 1). This latter pattern suggests incomplete dominance.

In the second set of crosses, the mean emission rates of Z7-12 : Ac for females from the P_1 and P_2 families were 47.81 and 40.33 ng/10 min, respectively (Figure 2a). Emission rates of Z7-12 : Ac from F_1, F_2, and backcross

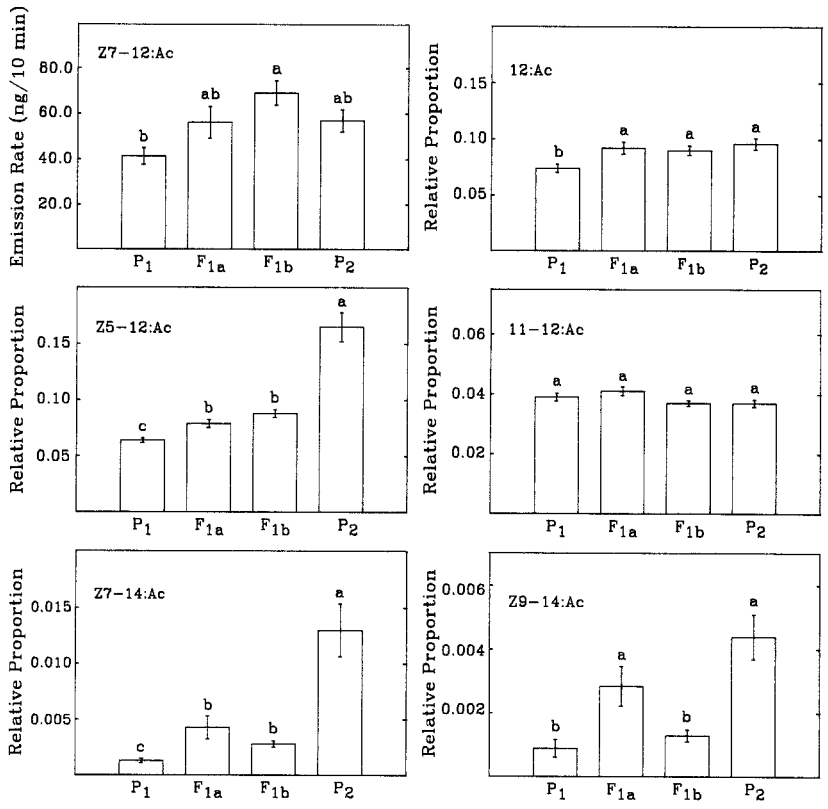


FIG. 1. Mean emission rate (±SE) of the major pheromone component, Z7-12:Ac, and mean relative proportions (±SE) of minor components emitted by P_1, P_2, and F_1 hybrid females during 10-min collection periods. For any component, means sharing the same letter do not differ at $\alpha = 0.05$, Sidak's multiple comparison test preceded by one-way ANOVA. Relative proportions were log-transformed before statistical analysis.

females were similar to each other but higher than for females from the parental lines.

The pattern of the relative proportions of minor components emitted by P_1, P_2, and F_1 hybrid females was similar to that found in the initial crosses. P_2 females emitted higher proportions of Z5-12:Ac, Z7-14:Ac, and Z9-14:Ac than did P_1 females, and F_1 relative proportions were intermediate (Figure 3), whereas differences between parental lines and F_1 hybrids in the proportion of 12:Ac and 11-12:Ac were not apparent (Figure 2b). Moreover, the proportion of 12:Ac and 11-12:Ac in volatiles emitted by F_2, BC_1, and BC_2 females were

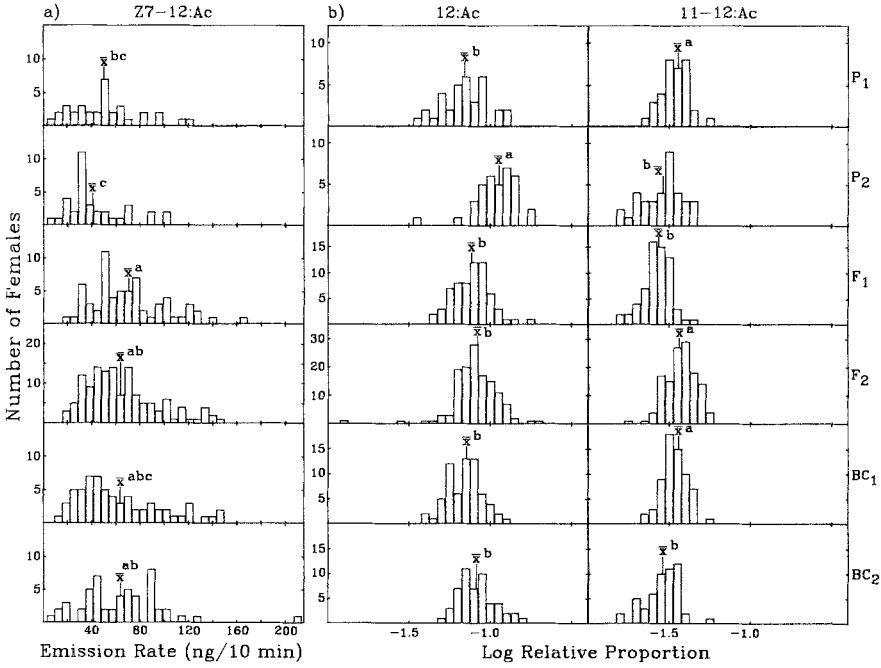


FIG. 2. (a) Frequency distributions of the emission rate of Z7–12:Ac emitted by P_1, P_2, F_1, F_2, and backcrosses (BC_1 and BC_2) during 10-min collection periods. Data are pooled from families listed in Table 1. Means ($\bar{x}$) are indicated. (b) Frequency distributions of the relative proportions (log transformed) of 12:Ac and 11–12:Ac emitted by females from the above crosses. For any component, means sharing the same letter do not differ at $\alpha = 0.05$, Sidak's multiple comparison test preceded by one-way ANOVA. Relative proportions were log-transformed before statistical analysis.

not clearly distinguishable from parental lines (Figure 2b). However, inheritance patterns for the remaining components suggest a genetic basis for the inheritance of pheromone blend.

The inheritance patterns for proportions of Z5–12:Ac, Z7–14:Ac, and Z9–14:Ac produced by F_2 and backcross females suggest that pheromone blend is most likely under polygenic control. Phenotypic segregation into categories characteristic of a trait controlled by a single autosomal or sex-linked gene is not apparent in the emission patterns (Figures 3 and 4). However, given the subtle differences between the P_1 and P_2 lines and the possibility that expected ratios in the F_1, F_2, and backcross generations might not be detectable by visual inspection, we tested the monogenic hypothesis by generating frequency distributions for Z5–12:Ac, Z7–14:Ac, and Z9–14:Ac that would be expected if

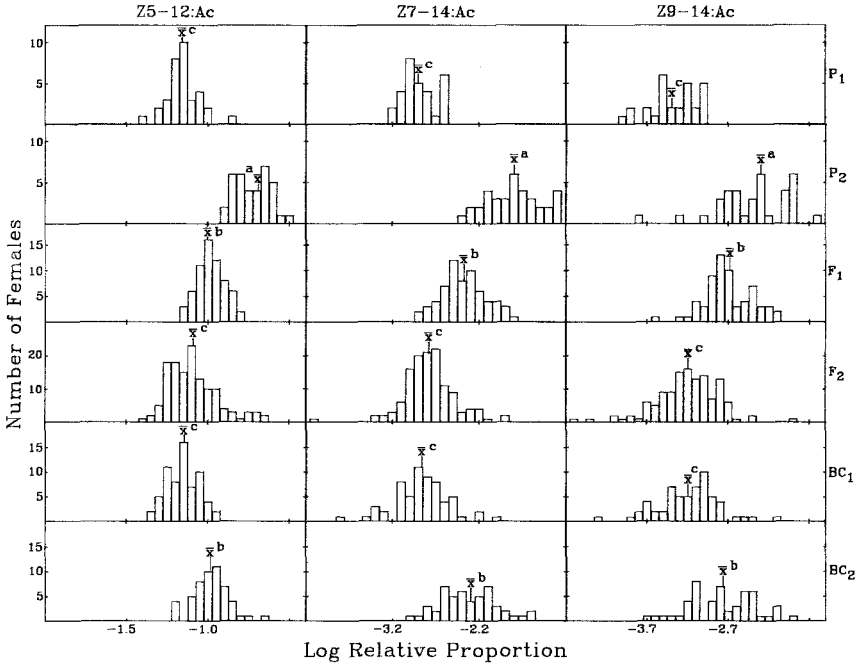


FIG. 3. Frequency distribution of the relative proportions (log transformed) of Z5–12:Ac, Z7–14:Ac, and Z9–14:Ac emitted by P_1, P_2, F_1, F_2, and backcrosses BC_1 and BC_2 females during 10-min collection periods. Means are indicated. For any component, means sharing the same letter do not differ at $\alpha = 0.05$, Sidak's multiple comparison test preceded by one-way ANOVA on log-transformed data.

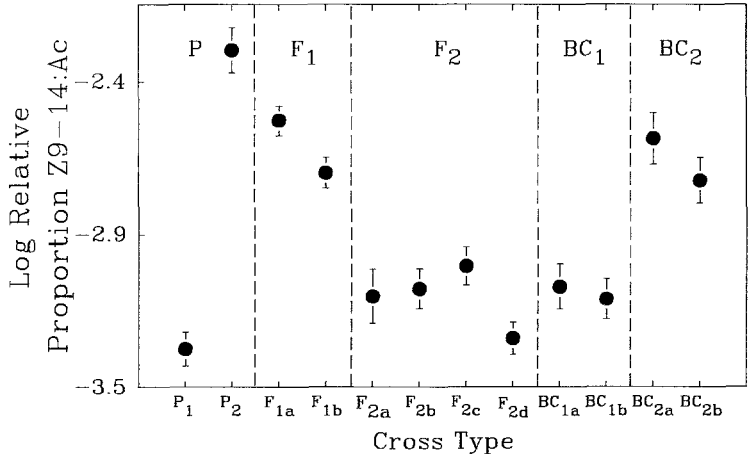


FIG. 4. Mean relative proportion (±SE) of Z9–14:Ac emitted by females during 10-min collection periods. Pooled data for these families are found in Figure 3. The parentage of each cross type is listed in Table 1.

each were under the control of a single autosomal or sex-linked gene. These expected distributions were then compared to those actually obtained in the F_1, F_2, and backcross generations.

To test the single autosomal gene hypothesis, expected frequency distributions were generated by pooling frequency data obtained for both parental types with the F_1 generation. Under this hypothesis the expected phenotypic ratio of $P_1 : F_1 : P_2$ in the F_2 generation is 1 : 2 : 1, respectively. Thus, frequency categories were weighted to conform to this expected ratio. The resulting trimodal frequency distribution then was compared to the actual F_2 distribution using the Kolmogorov-Smirnov (K-S) two-sample test (Siegel, 1956). For each of the pheromone components the observed frequency distribution was significantly different than the expected ($\chi^2 = 39.6$, $P < 0.0001$; $\chi^2 = 50.1$, $P < 0.0001$; $\chi^2 = 37.6$, $P < 0.0001$ for Z5–12 : Ac, Z7–14 : Ac, and Z9–14 : Ac, respectively). Frequency distributions of BC_1 and BC_2 females provide further evidence against the single autosomal gene hypothesis. Following a procedure similar to that described above for the F_2 data, weighted bimodal distributions (1 : 1; $F_1 : P_1$) were generated and then compared to the actual BC_1 frequency distribution. For each of the three components, the observed frequency distribution was significantly different than the expected bimodal (1 : 1; F_1 : P) distribution (K-S test; $\chi^2 = 6.6$, $P < 0.05$; $\chi^2 = 8.3$, $P < 0.05$; $\chi^2 = 10.8$, $P < 0.01$; for Z5–12 : Ac, Z7–14 : Ac, and Z9–14 : Ac, respectively). Similar results were obtained from the BC_2 crosses. These results support the polygenic hypothesis.

The single sex-linked gene hypothesis was tested following procedures similar to those described above. This hypothesis predicts that F_1 and backcross females should be identical to females of the paternal line from which they were derived. However, the means and frequency distributions of log relative proportions of Z5–12 : Ac, Z7–12 : Ac, and Z9–14 : Ac found for F_{1a} and F_{1b} females were intermediate to both parental lines (e.g., Z9–14 : Ac, Figure 4). For each of these pheromone components the frequency distribution of P_1 females was significantly different than F_{1b} females, as was the frequency distribution of P_2 females when compared to F_{1a} females (K-S test of P_1 vs F_{1b}; $\chi^2 = 24.4$, $P < 0.0001$; $\chi^2 = 27.0$, $P < 0.0001$; $\chi^2 = 35.9$, $P < 0.0001$; K-S test of P_2 vs F_{1a}; $\chi^2 = 36.3$, $P < 0.0001$; $\chi^2 = 20.2$, $P < 0.0001$; $\chi^2 = 7.8$, $P < 0.05$; for Z5–12 : Ac, Z7–14 : Ac, and Z9–14 : Ac, respectively. Similarly, females from each backcross family were intermediate to and significantly different than parental lines. Thus, emission patterns of Z5–12 : Ac, Z7–14 : Ac, and Z9–14 : Ac from F_1 and backcross females provides evidence that pheromone blend is not controlled by a single sex-linked gene.

Although not conclusive, our data suggest that one or more of the genes that control pheromone blend may be sex-linked. In the initial experiment, the relative proportion of Z9–14 : Ac found in F_1 females was closer to that for

females from the paternal line than females from the maternal line (Figure 1). This pattern suggests the involvement of one or more sex-linked genes with one or more autosomal genes. Some of the results from the second experiment supported this hypothesis when the relative proportions of *Z*9–14 : Ac for pooled families (Figure 3) were examined for each family separately (Figure 4). The relative proportions of *Z*9–14 : Ac found in F_1 and backcross females were closer to the paternal line (P_1 or P_2) from which they were derived. However, these results must be interpreted with caution because of the limited number of families that were examined.

Evidence presented here suggests that subtle differences in the cabbage looper pheromone blend are controlled by genes that sort independently, thus opening the possibility that the differences between the P_1 and P_2 colonies may have accumulated gradually. The P_1 colony has been maintained in laboratory culture for more than 100 generations and appears to have diverged from the typical blend ratio of pheromone components observed in field populations (Haynes and Hunt, 1990a). It appears likely that the genetic divergence that underlies the phenotypic changes in the pheromonal blend ratio may be explained by genetic drift, in the absence of gene flow from wild populations and with the relaxation of stabilizing selection imposed by long-range communication between the sexes. However, the possibility of some unknown directional selection pressure in the laboratory population cannot be discounted.

Genetic factors that influence the pheromone communication system of *T. ni* may provide insights into the process of speciation. The present study suggests that small incremental changes can result in divergence in the pheromone blend released by females. Similarly, the pheromone blend of the pink bollworm moth, *Pectinophora gossypiella* (Saunders), diverged gradually when artificial directional selection was imposed (Collins and Cardé, 1989). In contrast, in the red-banded leafroller, *Argyrotaenia velutinana*, an attempt to select for a higher percentage of the (*E*)-11-tetradecenyl acetate isomer over many generations did not result in a shift in blend ratios beyond the natural range variation (Roelofs et al., 1986). In fact, the modest changes that did result from this selection could not be sustained when individuals from different lines were crossed, which may suggest the existence of additional genetic factors that, under normal circumstances, may limit the ability of individuals to adapt their pheromone blend to selection pressure. More direct evidence of genetic linkage that might limit the potential to respond to selection comes from the multimodal sex communication system of the sulfur butterfly, *Colias eurytheme* Boisduval (Grula and Taylor, 1979). Genetic control of the male-produced pheromone (13-methyl heptacosane) and the UV reflectance pattern (a visual signal in courtship) are tightly linked on the *X* chromosome. Complex genetic changes may be necessary for significant phenotypic changes to occur in communication systems that are rigidly canalized.

The potential for adaptations of pheromone blend of our laboratory colony to selection does not appear to be limited by rigid canalization of the genetic factors that control it. In fact, a single gene mutation that results in a 30-fold increase in the abundance of a minor pheromone component (Z9–14 : Ac) and near absence of another minor component (Z5–12 : Ac) has been documented in a few individuals in the colony (Haynes and Hunt, 1990b). Within the cabbage looper there appears to be the genetic potential for gradual accumulation of polygenic changes in the pheromone blend, as documented in this paper, and for dramatic changes caused by a single gene mutation. Additional experiments imposing directional selection on male moths may help to determine whether abrupt or gradual changes in the pheromone blend are more likely to lead to the species-specific pheromone blends that are common in natural systems.

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RESPONSES BY DOMESTIC CATS (*Felis catus*) TO SNAKE SCENT GLAND SECRETIONS

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Abstract—The scent gland secretions of snakes are thought to repel predators, but few predator species have been tested for responses to these exudates. Domestic cats (*Felis catus*) were tested for responses to scent gland secretions of the gray rat snake (*Elaphe obsoleta*), or to choloroform extracts of them, applied to filter paper or food. More cats salivated or rubbed on filter papers treated with scent gland secretions than on control papers. Scent gland exudates elicited rubbing and pawing in cats more frequently than did chemicals from a shed snake skin. Cats offered food pieces treated either with water or with scent gland secretions ate fewer of the latter; this result is consistent with the hypothesis that scent gland secretions deter feeding.

Key Words—Domestic cat, *Felis catus*, rat snake, *Elaphe obsoleta*, snake scent glands, feeding deterrence.

INTRODUCTION

Snake scent glands generally are thought to produce secretions to repel predators (e.g., Greene, 1987). Few attempts, however, have been made to test the antipredator function of scent gland secretions, and only a couple of accounts describe responses to them by mammalian predators. An informal test indicated that several carnivores gave "aversionary" responses to food treated with the exudates of the kingsnake (*Lampropeltis getulus*) (Price and LaPointe, 1981). An investigation of canids' responses to the secretions of the western diamondback rattlesnake (*Crotalus atrox*) demonstrated that coyotes (*Canis latrans*) were attracted to and rubbed on these materials and that dogs (*Canis familiaris*)

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mouthed and exhibited urination postures to them (Weldon and Fagre, 1989). The defensive function of scent gland secretions vis-à-vis these carnivores was uncertain with these results.

We report here reactions by domestic cats (*Felis catus*) to whole and solvent-extracted scent gland secretions of the gray rat snake (*Elaphe obsoleta*; Colubridae), a nonvenomous North American species. This study documents salivation and other responses by felids to scent gland secretions, and it provides evidence for the possible feeding deterrent properties of these exudates.

METHODS AND MATERIALS

Experiment I. The purpose of this experiment was to determine cats' reactions to raw scent gland secretions. Approximately 4.2 g of scent gland secretions were obtained from three adult rat snakes by manually pressing the base of the tail and collecting the secretions in a vial. The secretions were transported on ice and stored at −90°C. The secretions were reconstituted by adding 2.5 ml of water. Whatman filter papers (W&R Balston Limited, England, (7 cm diameter)) were treated with 25 mg of reconstituted secretions or, for controls, three drops of water. The papers were allowed to dry under a hood for 2 min. They were then placed into plastic Petri dishes and stored on ice.

Fifteen adult (> 1-year-old) domestic cats were tested. The cats were housed individually in 64 × 121 × 121-cm cages equipped with a perch and litter box. Subjects were fed approximately 85 g of dry and canned food twice a day at 0800 and 2000 hr. Water was provided ad libitum. All but one subject had been raised indoors; this was the only individual that might have encountered snakes. Cats had been exposed to the scent gland secretions of the western diamondback rattlesnake six months prior to this experiment during preliminary tests.

Each filter paper was taped to a 7.6 × 17.2-cm metal plate, which was then placed on the floor of each cat's home cage for 3 min; separate plates were used for secretion- and water-treated papers. The following behaviors were scored: mouthing (licking, biting, or eating paper), salivation (evidenced by droplets on filter paper), pawing at paper, and head and body rubbing against filter paper (Figure 1) and cage walls. An observer was positioned within 1 m of the front of each cage; the same observer was involved in all experiments.

Control and secretion-treated papers were presented in a counterbalanced design, with 10–15 min elapsing between presentations. Testing was done between 1000 and 1400 hr.

Nine cats responded during control or experimental presentations (Table 1). The McNemar test failed to detect significant differences between treatments, although the tendency of more cats to rub during presentations of secretion-treated papers approached significance ($\chi^2 = 3.2$, $0.10 > P > 0.05$).



FIG. 1. A cat vigorously rubs its head against a filter paper treated with scent gland secretions from a rat snake.

Experiment II. The purpose of this experiment was to determine cats' reactions to a chloroform ($CHCl_3$) extract of scent gland secretions. Scent gland secretions were collected from a rat snake, as described. Approximately 2 ml of $CHCl_3$ were added to the collecting vial, which was transported on ice and stored at −90°C. Secretions were extracted by adding several milliliters of $CHCl_3$, shaking the vial, and allowing its contents to settle for several minutes. The lower $CHCl_3$ layer was then drawn off with a pipet. This procedure was repeated until 25 ml of $CHCl_3$ extract were obtained. The concentration of materials in the extract was determined by transferring a 2-ml aliquot to a tared vial. The $CHCl_3$ was removed by placing the vial in a warm water bath under N_2. The weight of the $CHCl_3$-soluble material obtained after desiccation in vacuo for 2 hr indicated a 3.4 mg/ml solution.

TABLE 1. RESPONSES BY 15 CATS TO FILTER PAPERS TREATED WITH WATER OR SCENT GLAND SECRETIONS FROM RAT SNAKE

Treatment	Mouthing	Salivating	Pawing	Rubbing
Water	2	0	5	0
Scent gland	1	3	3	5

Five drops of scent gland secretion extract or $CHCl_3$ were applied to filter papers (5.5 cm diameter). The papers were allowed to dry under a hood for 2 min and then were placed into plastic Petri dishes and stored on ice.

Fourteen cats were tested. They were maintained and tested as described for the previous experiment. Testing was done between 1000 and 1200 hr.

Eleven cats responded during control or experimental presentations (Table 2). The McNemar test detected significantly more cats salivating to extract-treated papers ($\chi^2 = 5.1$, $P < 0.05$). No other significant differences were detected.

Experiment III. The purpose of this experiment was to compare cats' reactions to snake scent gland and epidermal lipids. A naturally shed skin (7.3 g) of the rat snake donating scent gland secretions in the previous experiment was obtained. The skin was cut into pieces approximately 5 cm long and extracted twice with 300 ml of $CHCl_3$ at 40°C for 2 hr. The extracts were combined, filtered, and reduced to approximately 25 ml by rotoevaporation. The concentration of materials in the extract was determined by transferring a 2-ml aliquot to a tared vial. The $CHCl_3$ was removed by placing the vial in a warm water bath under N_2. The weight of the $CHCl_3$-soluble material obtained after desiccation in vacuo for 2 hr indicated a 38.5 mg/ml solution.

Five drops of skin extract or 15 drops of scent gland secretion extract were applied to filter papers (7 cm diameter). The papers were allowed to dry under a hood for 2 min. They were then placed into plastic dishes and stored on ice.

Twenty-four cats were tested. Fourteen cats were maintained individually, as described. Ten adult cats (histories unknown) were housed in groups of two to four. The group-housed cats were transferred to individual 64 × 64 × 64-cm cages and allowed 30 min to acclimate before filter papers were presented. They were given water ad libitum and fed dry food once a day at 0800 hr.

Cats were tested as described for the previous experiments. Testing was done between 0900 and 1200 hr.

Seventeen cats responded during presentations of skin or scent gland extracts (Table 3). The McNemar test detected significantly more cats rubbing ($\chi^2 = 7.2$, $P \leq 0.05$) and pawing ($\chi^2 = 4.9$, $P \leq 0.05$) to scent gland-treated papers; the greater number of cats salivating to scent gland papers approached

TABLE 2. RESPONSES BY 14 CATS TO FILTER PAPERS TREATED WITH CHLOROFORM OR CHLOROFORM EXTRACT OF SCENT GLAND SECRETIONS FROM RAT SNAKE

Treatment	Mouthing	Salivating	Pawing	Rubbing
Chloroform	3	0	6	2
Scent gland extract	2	7	6	3

TABLE 3. RESPONSES OF 24 CATS TO FILTER PAPERS TREATED WITH CHLOROFORM EXTRACTS OF SCENT GLAND SECRETION OR SHED SKIN FROM RAT SNAKE

Treatment	Mouthing	Salivating	Pawing	Rubbing
Shed skin extract	2	6	8	2
Scent gland extract	4	10	14	7

significance ($\chi^2 = 3.1$, $0.10 \geq P \geq 0.05$). No significant difference was detected with respect to mouthing.

Experiment IV. The purpose of this experiment was to determine cats' reactions to food treated with raw scent gland secretions. Scent gland secretions, pooled in a vial from five adult rat snakes, were transported on ice and stored at −90°C. The secretions were reconstituted by adding 0.5 ml of water.

Cats were presented with pieces (ca. 38 mg each) of cat food (Tender Vittles, Ralston Purina, Missouri) in two plastic Petri dishes attached to a 27 × 15-cm tray; the edges of the dishes were 3.7 cm apart. Two food pieces, each treated with three drops of water, were placed into one dish, and two pieces, each treated with 8 mg of reconstituted scent gland secretions, were placed into the other dish. A tray was placed with a cat for 3 min. Cats were scored for their tendency to eat one or more food pieces.

Fourteen cats, maintained individually as described, were tested. All had been offered their normal daily ration of food at 0800 hr. Testing was done between 1100 and 1400 hr.

Nine cats ate water-treated food pieces, three ate secretion-treated food pieces. The McNemar test detected significantly fewer cats eating secretion-treated food pieces ($\chi^2 = 4.1$, $P \leq 0.05$). No other significant differences were detected.

DISCUSSION

Observations on gray foxes (*Urocyon cinereoargenteus*), bobcats (*Lynx rufus*), and other North American carnivores suggested that the scent gland secretions of the kingsnake deter feeding (Price and LaPointe, 1981). Evidence of repellency, however, was not observed in canids tested with extracts of the scent gland secretions of the western diamondback rattlesnake (Weldon and Fagre, 1989). These exudates, treated with or extracted in methylene chloride, were visited by coyotes at scent stations in the field more often than were controls, and, in two experiments, with a frequency not significantly different from that shown to a commercially available carnivore attractant. Evidence of rub-

bing on scent gland extracts also was observed. Domestic dogs exhibited urination postures (or urination) to scent gland extracts, and they mouthed filter papers treated with these materials more than those treated with solvent alone.

In our study, chloroform-soluble scent gland compounds elicited salivation in domestic cats. This response to snakes has been observed in other carnivores. Burton (1976) reported that a domestic dog salivated during an encounter with a grass snake (*Natrix* sp.), and Kano (1976) observed salivation by bears (*Ursus arctos*) when presented with snake carcasses (species not stated) or with a rope that had been kept with snakes. The anatomical source of substances to which these carnivores responded is unclear. Ridley (cited in Smith, 1938) reported that a dog foamed at the mouth after seizing a Malayan snake (*Macropisthodon rhodomelas*) and quickly dropped it; this presumably was elicited by secretions from the nuchodorsal glands, which have been described only in several Asian genera of natricine snakes.

Our experiments indicate that scent gland secretions elicit rubbing in cats and that scent gland compounds are more potent in eliciting this response than are those from a shed skin. In a preliminary study, cats rubbed against the secretions of the western diamondback rattlesnake, a crotaline, indicating that this is a general response to scent gland products. These results, and those of Weldon and Fagre (1989) with canids, suggest that rubbing is a widespread and possibly ancient carnivore response to snakes. Siberian chipmunks (*Eutamias sibiricus asiaticus*) also rub on scent gland- and other snake-derived chemicals, applying these materials to their fur (Kobayashi and Watanabe, 1981). This behavior is thought to disguise chipmunks chemically vis-à-vis chemosensory-oriented predators. Other mammals may derive similar benefits by appropriating snake scent gland exudates.

Salivation and head-rubbing are performed by many felids as scent-marking and anointing patterns (Wemmer and Scow, 1977). These responses are elicited by strong novel odors, such as those emanating from carrion, vomit, the feces of strange animals, and catnip (Wemmer and Scow, 1977). The active compounds in most of these materials are not known, although a number of behavioral releasers, including lactones and other cyclic compounds, have been isolated from plants (Tucker and Tucker, 1988). Chemicals similar to these plant products have not been isolated from snake scent gland secretions (Weldon et al., 1990, and references therein). Compounds known to occur in these exudates need to be tested for behavioral activity with felids.

The canids in Weldon and Fagre's (1989) investigation were not tested with scent gland secretion-tainted food, thus the palatability of these substances was not determined. Our study shows that cats are less inclined to eat secretion-treated than water-treated food pieces when these items are offered simultaneously, thus providing evidence consistent with an antipredator function for scent gland exudates. Whether these secretions were unacceptable due to the

novelty of the olfactory or gustatory cues they imparted is unclear, but shear novelty could elicit a reluctance to ingest and therefore constitute a defense mechanism.

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URINARY CHEMOSIGNALS, REPRODUCTION, AND POPULATION SIZE FOR HOUSE MICE (*Mus domesticus*) LIVING IN FIELD ENCLOSURES

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Abstract—The effects of urinary chemosignals influencing reproduction and puberty were studied in free-living house mice maintained in six 0.1-ha outdoor enclosures. Two enclosures were provided with urine and soiled bedding from male mice, two with urine and soiled bedding from group-caged female mice, and two with water and clean bedding as a control. Reproduction, puberty, recruitment, and population size all were affected by the urine treatments. Overall, populations reached significantly greater numbers in the enclosures treated with male urine and soiled bedding, intermediate levels with the water and clean bedding treatment, and were lowest for enclosures treated with urine and soiled bedding from grouped females. The population differences resulted from urinary chemosignal effects on adult female reproduction and female puberty; in general, females were in higher states of reproductive condition, more pregnancies occurred, and young females attained puberty earlier in male-treated enclosures and later in female-treated enclosures than in control enclosures. Urinary chemosignals that affect endocrine physiology and related reproductive processes may be one mechanism by which various factors influence population size in house mice.

Key Words—Urinary chemosignals, reproduction, *Mus domesticus*, populations, recruitment, puberty, sex ratio.

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INTRODUCTION

Urinary chemosignals affect reproduction in adult female rodents (McClintock, 1983) and puberty in young females (Vandenbergh, 1983; Drickamer, 1986a; Vandenbergh and Coppola, 1986). Urine from male mice (Whitten, 1958) and from pregnant or lactating females (Hoover and Drickamer, 1978) enhances reproductive cycles of adult females, whereas urine from grouped females results in delays in reproduction (Whitten, 1959, 1966). Urine from males (Vandenbergh, 1969), from pregnant or lactating females (Drickamer and Hoover, 1979), and urine from females in estrus (Drickamer, 1986b) all accelerate puberty in young females, whereas urine from females caged in groups results in delays in puberty (Vandenbergh et al., 1972; Drickamer, 1977). These chemosignal effects have been demonstrated in wild stocks of house mice maintained in the laboratory (Drickamer, 1979, 1984). In addition, stocks of wild house mice released on cloverleaf islands of highways produce the urinary chemosignals (Massey and Vandenbergh, 1980, 1981; Coppola and Vandenbergh, 1987) as determined by a laboratory bioassay using urine collected from free-living mice.

Urinary chemosignals also influence reproduction in other species of small rodents. For example, studies on *Peromyscus leucopus* (Haigh et al., 1985; Haigh, 1987), *P. maniculatus* (Terman, 1980, 1987), *Microtus ochrogaster* (Hasler and Conaway, 1973; Hasler and Nalbandov, 1974), and *Dicrostonyx groenlandicus* (Hasler and Banks, 1975a, b) provide evidence that urinary chemosignals influence reproductive processes in these animals. Not all of the effects observed in *Mus* occur in the same fashion in the other rodents. For example, in *P. maniculatus bairdi*, Terman (1980) and Lombardo and Terman (1980) reported that urine from adult females did not inhibit sexual development in young females, whereas Lombardi and Whitsett (1980) reported that the urine of adult females of this species did inhibit sexual development of young females. On the other hand, there are some clear similarities between the various rodents that have been investigated with respect to chemosignal effects on reproduction. For example, in *P. leucopus*, an airborne chemosignal from adult females inhibits its reproduction in young females (Haigh et al. 1985), and for *D. groenlandicus* the presence of an adult male stimulates the estrous cycles of adult females (Hasler and Banks, 1975b).

Urinary chemosignals that enhance reproduction in young and adult female house mice should result in faster-growing, larger populations because generation time for young animals is shortened and the percentage of adult animals reproducing is higher. In contrast, urinary chemosignals that delay reproduction and puberty in adult and young mice, respectively, should result in longer generation times for juveniles and lower rates of pregnancy for adults. This could lead to either a stable or declining population. To date, no one has tested the

effects of urinary chemosignals influencing puberty and reproducton in wild mice living in nature. We tested the effects of urinary chemosignals on house mouse populations living in outdoor enclosures to ascertain the validity of the foregoing hypotheses regarding urinary chemosignal influences on reproduction, puberty, and population size.

METHODS AND MATERIALS

Enclosures. Six enclosures located at the Touch-of-Nature outdoor education and research facility of Southern Illinois University at Carbondale were used for the study. The enclosures are located in an abandoned 1.5-ha horse pasture containing grasses and herbs (broom sedge, *Andropogon virginicus*; little blue stem, *Andropgon scoparius*; goldenrod *Solidago* spp.; fescue, *Festuca myuros* and *F. octaflora*; common milkweed, *Asclepias syriaca*; common garlic, *Allium canadense*; thistle, *Crisium vulgare* and *C. discolor*, and a variety of wild flowers). The enclosures are rectangular, 40 × 25 m each, with an inside area of 0.1 ha. They are constructed of 1-m high 0.6-cm mesh galvanized hardware cloth with 50 cm buried into the ground and 50 cm above ground attached every 1.3 m to steel fenceposts. Aluminum flashing, 40 cm high, is located above the hardware cloth and is attached to the fenceposts and bolted to the top of the hardware cloth every 10–12 cm. Aluminum pieces (30 × 30 cm) attached to the aluminum walls at the top of each fencepost prevent rodents from moving from enclosure to enclosure or escaping by climbing the posts, and they prevent entry by animals from outside the enclosure. A 1.5-m-wide swath is kept mowed along the inside and outside of all walls of each enclosure to prevent the growth of vegetation that might permit mice to leave or enter the enclosures.

Within each enclosure are located four structures we call "mouse cities" (see King, 1983); these are positioned in a symmetrical pattern with the center of each city located 10 m from an end wall and 8 m from a side wall of the enclosure. Each city is constructed of 1-m-high aluminum flashing arranged in a 2-m-diameter circle. The aluminum side walls are buried 10 cm in the ground and are attached to eight wooden posts spaced equally around the inside wall of the city. The floor of each city consists of 0.6-cm mesh galvanized hardware cloth that is rolled up at the edges and bolted to the aluminum side walls. Each city has a roof consisting of two 1.3 × 2.6 m sheets of plywood edged with pine furring strips for stability and coated with polyurethane paint. The roof is supported from below by a column of cinder blocks (20 × 20 × 40 cm) located at the center of the city and is held down by cinder blocks placed on top of the plywood sheets. There are two entrances to each mouse city consisting of a 2.5-cm-diameter hole drilled in the aluminum wall 20 cm above ground level. Cin-

der blocks are positioned inside and outside the city at each entrance hole to provide access for the mice. Eight nesting cavities are provided inside each city: two each in the inside entrance blocks and four in the bottom two cinder blocks of the central tower. Access to these nesting places is provided by 2.2-cm holes drilled in the cinder blocks. A 1.5-m swath is kept mowed around each city to provide easier access to the city. Each mouse city is supplied with food and water in sufficient quantity such that it is never used up between the weekly checks described below. Each enclosure contains a 6 × 9 station trapping grid with 5 m between traps and with one Sherman folding aluminum live-trap at each station. A cotton Nestlet (Ancare Corp., Huntington, New York) is placed in each trap throughout the field season.

Start-up Procedures. Populations were started on April 3, 1989, with groups of three female and one male (all aged 90–165 days) placed into each city, providing a total of four males and 12 females in each enclosure. The wild stock house mice used for stocking the enclosures were first- and second-generation offspring of wild *Mus domesticus* trapped at Little Egypt Grain Co., Crab Orchard National Wildlife Refuge, Williamson County, Illinois. The mice were placed together in designated groups one week (March 27) prior to introduction into the cities. The cages were transferred from the laboratory to a covered outdoor location immediately after grouping where the mice were exposed to local ambient and photoperiod conditions. Each mouse was provided with a unique toe-clip and ear-punch number. The cities were closed by placing rubber stoppers in the exit holes at the time the mice were introduced. One week later, on April 10, the mouse cities were checked for the presence and health of the mice, and the corks were removed permitting all mice access to the entire enclosure. The mouse populations were allowed to grow freely for 30 weeks.

Treatments. To test our hypotheses, three treatments were used, each applied to two enclosures selected at random: (a) water and clean bedding as a control, (b) urine and soiled bedding from wild stock male house mice, and (c) urine and soiled bedding from grouped wild stock females. Wild stock mice producing the urine and soiled bedding were from the same stock used for providing mice to start the enclosures. They were 90–210 days of age at the time they were used for urine and bedding production. Males were housed 1–2/cage and females 4–6/cage. Twenty-four-hour urine samples were collected using metabolism cages (Maryland Plastics), diluted with three parts distilled water and refrigerated for up to six weeks until used in the enclosures (see Drickamer, 1982). Soiled bedding was collected at biweekly cage changes in the laboratory and was stored in 40-liter plastic bins until used in the field enclosures. Both urine and soiled bedding treatments were provided inside each mouse city. The two food dishes and water dish were placed in a plastic tray and surrounded by soiled or clean bedding as designated by the treatment condition for that enclo-

sure. Urine placed in perfume atomizers for spraying was again diluted with three parts water (making the final dilution 9:1, well within the effective range for both chemosignals tested; Drickamer, 1984). The diluted urine, or plain water for the control condition, was sprayed on the food dishes, water dish, soiled bedding surrounding the dishes, and at the entrances of all eight nesting cavities in each mouse city. The urine, water, and bedding treatments were provided during the weekly checks of the mouse cities described below. Thus the mice were exposed to urinary chemosignals, and possibly related odor cues, in a passive fashion as they went about their normal activities.

Weekly Procedures. Each Sunday we examined the interior of every city for mice and nests, replenished food and water supplies, replaced soiled bedding, and sprayed the urine solution on designated locations. The live traps were opened and baited with a pinch of rolled oats each Tuesday and Thursday between 1600 and 1800 hr. The traps were checked and closed on Wednesday and Friday mornings between 0600 and 0800 hr. For each mouse captured during city checks or trapping sessions, we noted the capture site, number, body weight, and reproductive condition and immediately released it at the capture site. New captures were given a toe-clip and ear-punch number and a permanent record was initiated. For the final three weeks of the study we used a combination of live-traps open each night and snap-traps to remove all mice from the enclosures. Trapping in the enclosures for three weeks in March 1990 resulted in no captures of any additional rodents.

Analyses. For females, we used a vaginal smear, body weight, palpation of the abdomen, and examination of the mammae to assign a value of 0–4 as a reproduction index: 0 = vagina closed, not pregnant or lactating; 1 = vagina open, not in estrus; 2 = vagina open, in estrus; 3 = pregnant; 4 = lactating. Only sexually mature females were assigned reproduction index values. The vaginal smears were made with a Pasteur pipet and examined in the field with a light microscope using the criteria of Vandenbergh (1969). Assessment of the interobserver reliability for evaluation of vaginal smears and assignment of reproduction index values resulted in values of $r = 0.85$ to $r = 0.95$. In addition, body weights taken from capture–recapture data on mice in the enclosures indicated no significant differences across treatments in growth rates for mice between 6.5 g and 16 g. These weights correspond to ages of 20–80 days, the period when puberty occurs. For males, we recorded whether the testes were scrotal or not; since no significant treatment effects were noted for either reproductive condition or age of first scrotal testes in males, we have omitted further data on this matter from our results and discussion.

For many of the analyses, the 30-week field season was divided into 10 three-week periods. Because we could capture and record all of the mice in the enclosures, we used an enumeration census. For data analysis we used ANOVA and chi-square tests (Steel and Torrie, 1960).

RESULTS

The enumeration population data (Figure 1) were subjected to a repeated-measures ANOVA, with treatments as the main factor and time periods as a repeated measure. Male-treated enclosures had significantly larger populations than female-treated enclosures; controls were intermediate and significantly different from both chemosignal treatments ($F = 27.66$; $df = 2,3$; $P < 0.001$). Peak numbers of about 50 mice were attained for male-treated enclosures in late summer, whereas female-treated enclosures peaked at about 20 mice and control enclosures at about 30 mice (Figure 1). A post-hoc analysis using Tukey's w procedure (Steel and Torrie, 1960) and $\alpha = 0.05$ was used to test for differences between the three treatments for each three-week period. There were no differences until the fourth time interval, at which time the male-treated enclosures had significantly larger populations than the other two treatments. Populations in male-treated enclosures remained larger for the remainder of the experiment. Populations in control enclosures were significantly larger than those in the female-treated enclosures at the sixth three-week period and for the last four periods. Overall, there was a significant time-periods effect ($F = 22.33$; $df = 9,27$; $P < 0.001$) as a result of increases of mice during summer months. A significant interaction between treatments and time periods ($F = 6.91$; $df = 18,27$; $P < 0.001$) can be attributed to shifts in population sizes during the first four three-week intervals (Figure 1).

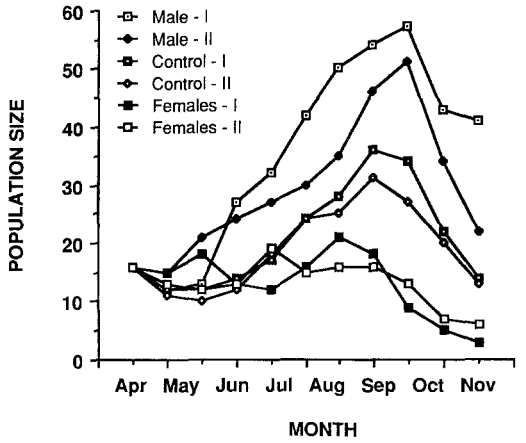


FIG. 1. Population size, for 10 three-week periods starting on April 10 and ending on November 5, for wild house mice living in field enclosures and provided with clean bedding and water (control) or soiled bedding and urine from either males or grouped females. Two replicate enclosures (I and II) were run for each treatment.

We defined recruitment as the number of first-time captures of young mice. Significantly more mice were recruited in male ($N = 154$) than in control ($N = 85$) or female-treated ($N = 49$) enclosures ($\chi^2 = 59.31$; $df = 2$; $P < 0.001$) and more mice were recruited in control than female-treated enclosures. Among the recruits, there was no overall significant sex ratio difference across the three treatments (Table 1(A); $\chi^2 = 5.50$; $df = 2$; $0.05 < P < 0.10$), but in the control enclosures there was a significant deviation from a 50:50 expected ratio with a bias toward female recruits ($\chi^2 = 8.58$; $df = 1$; $P < 0.01$). There was a significant shift in the sex ratio over the spring, summer, and fall (Table 1(B); $\chi^2 = 11.22$; $df = 2$; $P < 0.001$); slightly more males than females were recruited in the initial portion of the experiment (May and June). This ratio shifted to a slightly female-biased pattern of recruitment in the middle period (July and August) and was heavily female-biased in the final months (September and October).

There was a significant difference in the number of females attaining first estrus across the three treatments (Table 2; $\chi^2 = 31.46$; $df = 2$; $P < 0.001$). However, there was no difference in the proportion of females attaining puberty for the three treatments ($\chi^2 = 4.83$; $df = 2$; $0.05 < P < 0.10$). The weights of females in the male-treated enclosures differed significantly from those in the female-treated enclosures at the time we obtained the first estrus smear (Table 2; $F = 6.65$; $df = 2,70$; $P < 0.01$); females in the control enclosures were intermediate in mean body weight at the time of the first estrus smear.

A repeated-measures ANOVA was performed on the data from the reproduction index (see Conover and Iman, 1981, regarding ANOVA on ordinal

TABLE 1. SEX RATIOS FOR RECRUITS IN POPULATIONS OF HOUSE MICE INHABITING FIELD ENCLOSURES TREATED WITH ONE OF TWO URINE AND SOILED BEDDING REGIMENS OR WATER AND CLEAN BEDDING[a]

A. Recruitment—sex ratio by treatment Treatment	Males	Females
Male enclosures I and II	67 (65.8)	87 (88.2)
Control enclosures I and II	29 (36.3)	56 (48.7)
Female enclosures I and II	27 (20.9)	22 (28.1)
B. Recruitment—sex ratio by time periods Time Periods		
Periods 2–4 (May and June)	42 (32.5)	34 (43.5)
Periods 5–7 (July and August)	48 (44.8)	57 (60.2)
Periods 8–10 (September and October)	33 (45.7)	74 (61.3)

[a] A Sex ratios (expected values from χ^2) for the three treatments. B Sex ratios (expected values) for three clusters of (three-week time blocks during the experiment. Two enclosures were used for each treatment.

TABLE 2. NUMBER OF FEMALES ATTAINING PUBERTY AS MEASURED BY OBTAINING AN ESTRUS SMEAR (EXPECTED VALUES FROM χ^2) FOR HOUSE MICE IN FIELD ENCLOSURES WITH THREE DIFFERENT TREATMENT CONDITIONS AND TWO ENCLOSURES PER TREATMENT

Treatment	Attained first estrus	Did not attain first estrus	Body weight (g ± 1 SEM) at time of first estrus smear[a]
Male-treated enclosures I and II	48 (44)	5 (9)	15.0 ± 0.3^{A}
Female-treated enclosures I and II	20 (23)	8 (5)	16.2 ± 0.5^{AB}
Control enclosures I and II	9 (9)	2(2)	17.6 ± 1.0^{B}

[a] Those means in the column not marked by the same superscript letter are significantly different at the 0.05 level by Tukey's *w* procedure.

data). Overall, for male-treated enclosures the reproductive index (mean = 2.2; median = 2) was significantly higher than for control (mean = 1.6; median = 1) or female-treated (mean = 1.5; median = 1) enclosures ($F = 9.48$; $df = 2,3$; $P < 0.001$). A Tukey's *w* procedure revealed there were no differences between treatments for the reproductive index for any of the individual time periods. Reproductive condition varied significantly across time periods ($F = 4.67$; $df = 9,18$; $P < 0.001$); mice in all enclosures were in higher reproductive states in summer months than in spring or fall. The interaction term for treatments-by-time periods was not significant ($F = 0.94$; $df = 18,27$; $P > 0.50$).

We performed several analyses of pregnancy rates for mice in the enclosures. There were significantly more pregnancies in the male-treated enclosures (Table 3; $\chi^2 = 5.41$; $df = 2$; $P < 0.01$) than for the other two treatments, but there were no significant differences in the proportions of pregnancies for the three treatments ($\chi^2 = 3.49$; $df = 2$; $P > 0.10$). There were no significant differences between the three treatments with respect to the number of pregnancies per female for those mice that were used to start the enclosure populations (Table 3; $F = 1.31$; $df = 2,42$; $P > 0.10$). However, there were significant differences between the treatments with respect to the number of females born in the enclosures that achieved at least one pregnancy (Table 3; $\chi^2 = 5.53$; $df = 1$; $P < 0.025$).

TABLE 3. OCCURRENCE OF PREGNANCY (EXPECTED VALUES FROM χ^2) AND NUMBER OF PREGNANCIES IN FEMALES USED TO START ENCLOSURE POPULATIONS AND NUMBER OF FEMALES BORN IN ENCLOSURES THAT BECAME PREGNANT (EXPECTED VALUES).

Treatment	Pregnancy in starter females		No. pregnancies (mean ± 1 SEM)	Young females achieving pregnancy[a]	
	Yes	No		No	Yes
Male-treated I and II	28 (22)	7 (13)	2.4 ± 0.3	10 (6.6)	6 (9.4)
Control I and II	14 (15)	10 (9)	2.1 ± 0.4	2 (7.4)	9 (10.6)
Female-treated I and II	17 (15)	7 (9)	1.7 ± 0.2	2	5

[a] The pregnancy values were combined for control and female-treated conditions because of the low frequencies.

DISCUSSION

The major conclusion from our experiment is that urinary chemosignals which, under laboratory conditions affect adult reproduction and puberty in juvenile house mice, have similar effects in populations of mice living in enclosures. Further, there are clear consequences of the effects on reproduction and puberty with respect to growth rates and sizes of the populations under different chemosignal treatment regimes. Populations in male-treated enclosures grew more rapidly and attained higher overall levels than populations in enclosures treated with water and clean bedding or with urine and soiled bedding from grouped females. Populations were smaller, for the latter portion of the test period, in enclosures treated with urine from grouped females than in enclosures treated with water. Urinary chemosignals may play a significant role in relation to regulation of numbers in populations of house mice.

What factors contribute to these differences in population size? Greater recruitment of new animals and the resulting larger populations in male-treated enclosures can be attributed to several interrelated events: (a) adult females were pregnant, lactating, or in estrus more frequently, (b) more females were born into the populations and more attained puberty, and (c) puberty was attained earlier than in the other two treatments. Smaller populations in female-treated enclosures relative to controls by late summer may be the result of the combined effects of: (a) fewer females attaining puberty in the female-treated enclosures,

and (b) some females attaining puberty at later ages in female-treated enclosures.

In addition to the variables that we measured, it is possible that some other factor(s) may have been acting to depress populations in female-treated enclosures. One such factor may be a phenomenon whereby females cannibalize litters produced by other females in the same cage or enclosure (P. Franks and W. Potts, personal communications). In our field enclosures, it is possible that similar cannibalism may have taken place. In female-treated enclosures, reproduction may have taken longer to reach some threshold, leading to sustained, successful reproduction. In male-treated enclosures, only one litter discovered shortly after birth was not later accounted for, whereas in female-treated enclosures five litters seen early in development were not found at a later time, at least three of which were definitely cannibalized. For control enclosures, three litters were seen early in development but not found later.

Our results suggest that urinary chemosignals may be a mechanism for mediating the effects of a variety of factors on reproduction. The primary proximate cues affecting the reproductive physiology of the mice are the various chemosignals encountered by the mice in their environment. Release of the various urinary chemosignals may be triggered by a number of social and environmental conditions (see Drickamer, 1986a), including population density, weather conditions, food/nutrition availability, aggression, etc. If we employ such logic concerning chemosignals and populations, then it is wise to be careful concerning the focus of natural selection (Cockburn, 1988). As the explanations that follow indicate, there are clearly pathways of interpretation concerning the evolution of the puberty-influencing chemosignals that are based on selection at the level of individual male and female mice.

For the urinary chemosignal from adult males, we posit that both the enhancement of reproductive condition in adult females and acceleration of puberty in young females resulting from contact with this chemosignal could lead to the availability of more potential mates. In fact, male mice deposit their urine differentially more frequently near odor cues from females (Drickamer, 1989a). Note that both young *P. leucopus* (Haigh, 1987) and young *M. domesticus* (Drickamer, 1989b) avoid odor cues from adult males prior to puberty. Adult *M. domesticus* shift their preference at about the time of puberty and begin to seek out odor cues from males (Drickamer, 1989b); they continue to do so as adults. Young females may avoid male cues because of negative consequences for individual fitness (Drickamer, 1988); more females than expected die earlier in their lives and have lower reproductive output to six months of age if puberty is accelerated when compared to females where puberty is delayed.

For interpretation of possible evolution concerning the urinary chemosig-

nal from grouped females that retards reproduction in adult females and delays puberty in young females, there are several possible scenarios (see Haigh, 1983, 1987). Why should adult or young mice respond, in terms of their reproductive physiology, to cues that slow down their reproduction? This is particularly true with reference to the short life-span of house mice (Berry, 1969; Berry et al., 1973). The release of a puberty-delaying chemosignal from females may be a by-product of ongoing metabolic activity (Drickamer, 1986a). Females do not appear to deposit their urine differentially with respect to cues from other females (Drickamer, 1989a), although they do leave urine more frequently near males. Delays in reproduction for adult females are difficult to explain; they may be responding physiologically to signals that delay estrous cycles because there is some selective advantage to waiting for more propitious conditions. If the release of reproduction-delaying signals occurs when conditions for reproduction are less than adequate, then individual females might gain in future reproduction by delaying somewhat their own reproduction. Alternatively, we could postulate that females respond physiologically to the delay signal from other females because of the chemical nature of the urinary cue and the present situation is in a state of flux. We may be examining a system in transition; females that are exposed to delay signals may still be evolving ways to detect the validity of the delay signals. To respond to such delay signals might retard reproduction to a point where the chances for successful production of progeny are greatly diminished. We would not expect this to be a stable system for very long; females should be evolving mechanisms to determine whether to respond to the delay chemosignal or ignore it.

For young females of *Mus* and *Peromsycus*, delays in puberty have been associated with dispersal (Bronson, 1979; Haigh, 1987). Such delays may or may not be related to avoidance of inbreeding (Haigh, 1987). Another hypothesis is that puberty delays provide a selective advantage for adult females relative to young females recruited into a population. When only a small proportion of females are going to even have the opportunity for reproduction (Goundie and Vessey, 1986; Haigh, 1987), it may be that evolution has favored a system whereby those females that are already cycling can delay the onset of cycles in younger mice. Another hypothesis is that by delaying puberty, females may enhance their chances for successful reproduction and may have larger litters (Skyrja, 1978; Haigh, 1987; Drickamer, 1988). Further work will be needed on *Mus* to determine which, if any, of these explanations best fits the observed pattern of puberty delay for this species.

Novotny and colleagues (Novotny et al., 1986; Jemiolo et al., 1987) have provided tentative chemical identification for urinary compounds that accelerate and delay puberty in female mice in the laboratory. It might be worthwhile to provide field tests of these compounds using an experimental design similar to

that employed here. We would expect that, in addition to the effects on female puberty, the synthesized compounds described from Novotny's laboratory should also influence reproductive condition in adult females.

We recorded a seasonal shift in the sex ratio of recruits that became part of the trappable population in the six enclosures. Similar results have been reported for *P. leucopus* (Goundie and Vessey, 1986). The findings for *Mus* may be due to a shift in the strategy by the dams. Putting out more sons in the spring may correlate with greater tendencies to disperse, whereas the female-biased ratio in fall may be a more conservative strategy with female progeny remaining with their mothers until at least the following spring. Alternatively, as Goundie and Vessey (1986) postulate, there may be a relationship between the condition of the reproducing females at the different seasons and the sex ratio of their progeny (Trivers and Willard, 1973). Further data will be needed to decide between these alternatives as well as other possible explanations for the seasonal shift in sex ratio for *Mus*.

It is worth noting that in an earlier study of house mice placed in enclosures of exactly the same size (0.1 ha) as those we used (Stueck and Barrett, 1978) and in a similar field setting, the population densities that were recorded, peaks of 20–30 mice per enclosure, are quite comparable to those that we obtained. Our male-treated enclosures produced population densities that were higher than those reported by Stueck and Barrett (1978) due possibly to the enhancement effects from chemosignals that we have already discussed. Neither the earlier study nor the present one involved measurements of what happens to such mouse populations during winter conditions. Such an extension might be useful for revealing additional elements of the population story for this species as well as the possibility of interactions of effects from weather conditions and urinary chemosignals.

Only additional laboratory and field studies of other rodent taxa will provide enough evidence to permit a fully comparative analysis of social factors affecting reproduction. To the degree that there are similarities between *Mus* and the other rodents, the experiments we reported on here may have significant meaning for the overall understanding of social control of reproduction in rodents and the consequences for population numbers.

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ELECTROANTENNOGRAPHIC RESPONSES DIFFERENTIATE SIBLING SPECIES OF DINGY CUTWORM COMPLEX, *Feltia jaculifera* (GN.) (LEPIDOPTERA: NOCTUIDAE)[1]

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Abstract—The dingy cutworm is one of the more common and widely distributed of North American noctuids. Determination of the antennal responses of field-collected male moths to a standard test blend of pheromone components shows that two pheromonal phenotypes of the dingy cutworm occur throughout the prairie region of western Canada and at least two others occur in eastern Canada. The pheromone types are morphologically indistinguishable and, so far as is known, have similar life histories and biology. The two prairie types are broadly sympatric with partially overlapping seasonal flight periods. Although hybrids can be easily produced in the laboratory, there is no evidence of hybridization in the field. The pheromone system of the dingy cutworm is apparently rather plastic, resulting in a mosaic of pheromone types (sibling species) that appear to be reproductively isolated but have not differentiated morphologically.

Key Words—*Feltia jaculifera*, *Feltia ducens*, dingy cutworm, Lepidoptera Noctuidae, sex pheromone, pheromone polymorphism, pheromonal phenotypes, electroantennograms, antennal responses, reproductive isolation, sibling species.

INTRODUCTION

The dingy cutworm has been widely known as *Feltia ducens*, but is now recognized as *Feltia jaculifera* (Gn.) (Franclemont and Todd, 1983). It is widely

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distributed in North America (Figure 1) and within much of its range is one of the most common noctuids (Rings and Arnold, 1976; Rings et al., 1975, 1978). Although often very abundant, particularly on native grasslands, it is only occasionally a destructive pest of agricultural crops (Beirne, 1971; Rings and Arnold, 1977). However, there are indications that the dingy cutworm may become a more important pest with the adoption of conservation tillage systems (Musick, 1987). In southern Alberta the only infestations of dingy cutworm in recent years have been in fall-planted cereals in no-till, continuously cropped fields. Because the dingy cutworm overwinters as early- to mid-instar larvae, it requires habitats having some fresh plant growth in both late fall and early spring.

Our initial attempts to identify the sex pheromone of the dingy cutworm yielded inexplicable results during the testing of candidate blends for attractancy in the field. Attempts to optimize a blend were contradicted by the attraction of male dingy cutworm moths to divergent blends. Subsequently, identification of the sex pheromone components in abdomen-tip extracts from

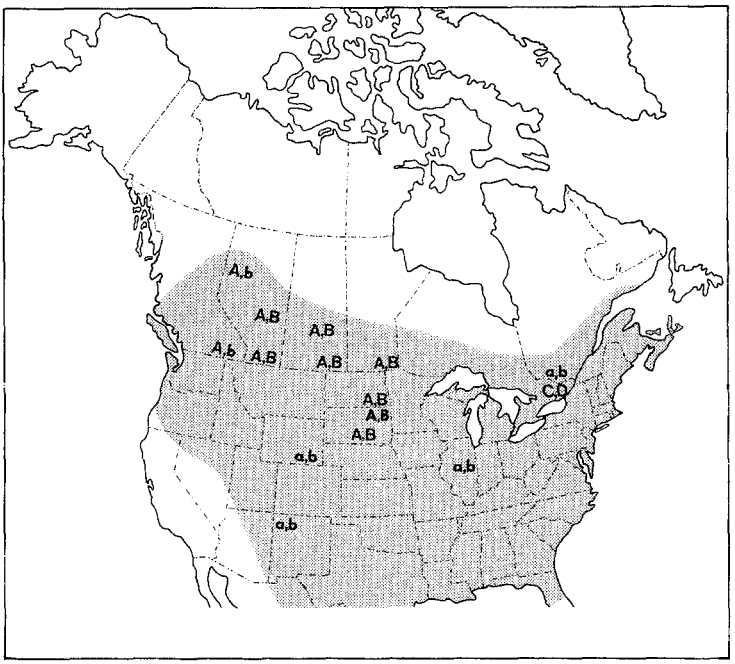


FIG. 1. Map showing distribution of *Feltia jaculifera* (Gn.), based on data from Rings and Arnold (1977) and specimens in the Canadian National Collection. The letters A–D indicate localities where the four pheromone types described in this paper are known to occur. Lower case a and b indicated localities where traps baited with the attractants for A and B types did not catch any *F. jaculifera* moths.

females of isofemale lines revealed that some lines produced sex pheromones with markedly different component ratios (Byers and Struble, 1990).

In this paper we describe the use of a capillary column gas chromatograph combined with an electroantennographic detector (GC-EAD) to determine the antennal responses of male dingy cutworm moths. The method facilitated the rapid determination of the antennal response profile and, hence the pheromone type of male moths obtained from various sources.

METHODS AND MATERIALS

Electroantennographic responses were determined by gas chromatography (GC) with a modified Varian model 2700 using both a flame ionization detector (FID) and an electroantennographic detector (EAD) simultaneously (Struble and Arn, 1984). A Supelcowax 10 fused silica column (30 m × 0.32 mm ID, film thickness 0.25 μm) was used for all analyses. Helium carrier gas linear velocity was 30 cm/sec and the temperature condition was 220°C isothermal.

Both antennae from each male moth were detached and placed in parallel across the electrodes of the EAD. The use of both antennae improved the magnitude and consistency of responses. The standard test blend consisted of Z7-12 : Ac, Z9-14 : Ac, and Z11-16 : Ac in a ratio of 1 : 1 : 1 in *n*-hexane at a concentration of 1 ng/μl each. Two microliters of the blend was split injected (10 : 1) for each run, and the effluent was split in a 2 : 1 ratio between the FID and EAD detectors. Responses were recorded with a dual-channel recorder. Antennal responses could be determined for six to seven moths per hour.

Male moths for EAD determinations were collected with light traps, while feeding on sunflowers, or in sex pheromone traps (design 1, Struble, 1983) baited with sex attractants or virgin females from isofemale lines. The sex attractant for type A was a 10 : 1 blend of Z7-12 : Ac and Z9-14 : Ac at 2.2 μg/septa and for type B a 1 : 1 : 2000 blend of Z7-12 : Ac, Z9-14 : Ac, and Z11-16 : Ac at 200 μg/septa (Byers and Struble, 1990). The bait females were from isofemale lines of known type reared on an artificial diet (Hinks and Byers, 1976). The isofemale lines of A and B originated from females collected at Lethbridge, Alberta, and those of C and D from females collected at Ottawa, Ontario.

Principal component analyses (Seal, 1964) were applied within each year for the Lethbridge data to determine whether the pheromone types could be differentiated on the basis of their EAD responses. The principal components are independent variates obtained from linear combinations of the EAD responses and are used to summarize the total variation in the original EAD responses. Principal component scores that cluster together indicate similarity in the antennal response pattern to the pheromone components. Multivariate

one-way analyses of variance were used to compare the mean vectors of EAD responses of groups comprised of pheromone types, methods of capture, sampling years, and locations. The analyses were carried out using SAS (1985) software.

The seasonal flight periods of types A and B were determined from daily catches in two pairs of pheromone traps located 1.5 km apart at Lethbridge. The numbers of moths caught were plotted against time with smoothing of the points by spline interpolation (SAS/GRAPH, 1985).

RESULTS

During the study, from 1985 to 1988, antennal responses were determined for 354 males caught in light traps (320 at Lethbridge and 34 at Ottawa), 20 collected while feeding on sunflowers (Lethbridge), 327 caught in pheromone traps baited with sex attractants for the two western types (216 at Lethbridge and 111 at other locations in western Canada), and 197 caught in pheromone traps baited with virgin females of the two western types (Lethbridge).

The principal component analyses of antennal response profiles of male moths from Lethbridge, whether caught at sex attractants, in the light trap, on sunflowers, or at virgin females, produced distinct clusters of the principal component scores (Figure 2), and the first two principal components accounted for more than 95% of the variation. It was clear, therefore, that all antennal response profiles of the male moths were of type A or B (Table 1, Figure 3). Males of one type were never caught at the sex attractant for, or females of, the other type. The sample from the light trap was obtained over the entire flight period of dingy cutworm moths and indicates that no other pheromone types occur at Lethbridge. Antennal responses of laboratory-reared male moths were always clearly of one or the other type.

Within a type and year, the mean vectors of the responses of moths collected by the various methods were similar. The variance–covariance matrices were similar as well ($P > 0.05$). This showed that the samples collected using the different methods were from the same population.

The antennal response profiles of a few type B moths showed a nearly equal response to both $Z7$–12 : Ac and $Z11$–16 : Ac. Initially, we thought these might be indicative of hybridization and introgression. However, similar response profiles were sometimes obtained from reared males of isofemale type B lines. In plots of the principal component scores, these atypical response profiles clustered with type B and there was always a clear gap between the A and B clusters. The antennal response profiles of F_1 laboratory hybrids clustered between the parental types.

Antennal responses of male moths caught with sex attractants at four other

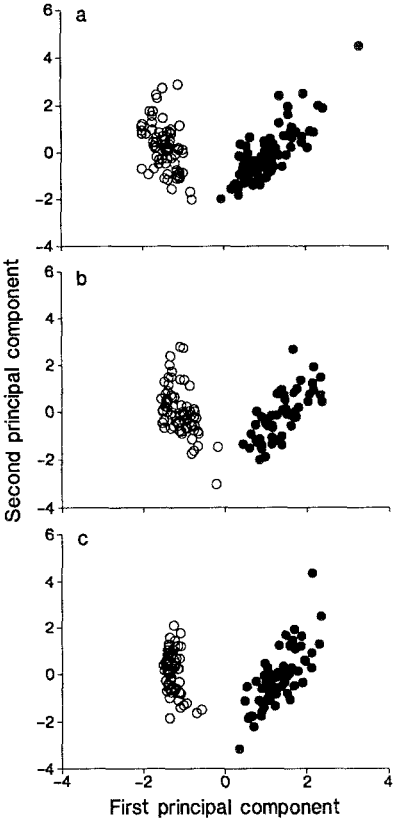


FIG. 2. Principal component analysis of antennal responses of male moths caught at Lethbridge, Alberta, with (a) sex attractants for types A and B, $N = 69$ and $N = 95$, 1985; (b) light trap, $N = 68$ and $N = 53$, 1985; (c) reared females of types A and B, $N = 69$ and $N = 68$, 1987. (o, type A; •, type B).

locations in Alberta and Saskatchewan were also clearly of A or B type (Table 1). However, multivariate analyses of variance indicated that there were significant differences ($P < 0.01$) between locations for both types, thus suggesting the possibility of geographical differentiation. The sample sizes were small and further investigation will be necessary to substantiate these differences.

Traps baited with the A and B sex attractants caught specimens of *F. jaculifera* at Winnipeg, Manitoba, Fargo, North Dakota, and several locations in eastern South Dakota (Figure 1). At Summerland, British Columbia, some *F. jaculifera* were caught at the A sex attractant but none at the B attractant. No live specimens for EAD determinations were obtained from these locations.

TABLE 1. ELECTROANTENNOGRAPHIC RESPONSES OF *Feltia jaculifera* (Gn). MALE MOTHS OF DIFFERENT PHEROMONE TYPES OR SOURCES TO TEST BLEND

	Pheromone type and source		Number tested	EAD response (millivolts, $\bar{X} \pm$ SD) Z7-12:Ac	Z9-14:Ac	Z11-16:Ac
I.	Samples of moths collected at Lethbridge by different methods					
A.	'A' attractant	1985	69	1.37 ± 0.38	0.19 ± 0.06	0.11 ± 0.04
	'A' attractant	1986	42	1.68 ± 0.20	0.29 ± 0.08	0.14 ± 0.06
	'A' females	1987	69	1.64 ± 0.21	0.31 ± 0.08	0.18 ± 0.05
	Light trap	1985	68	1.49 ± 0.34	0.21 ± 0.07	0.11 ± 0.04
	Light trap	1986	28	1.66 ± 0.19	0.27 ± 0.10	0.13 ± 0.04
	Light trap	1988	52	1.58 ± 0.19	0.37 ± 0.12	0.20 ± 0.09
	Sunflowers	1986	12	1.61 ± 0.32	0.24 ± 0.09	0.13 ± 0.06
B.	'B' attractant	1985	95	0.27 ± 0.11	0.21 ± 0.07	0.76 ± 0.26
	'B' attractant	1986	10	0.46 ± 0.18	0.35 ± 0.10	1.09 ± 0.31
	'B' females	1985	60	0.28 ± 0.15	0.22 ± 0.08	0.83 ± 0.34
	'B' females	1987	68	0.34 ± 0.15	0.31 ± 0.10	1.04 ± 0.34
	Light trap	1985	53	0.30 ± 0.12	0.21 ± 0.07	0.72 ± 0.28
	Light trap	1986	55	0.47 ± 0.16	0.32 ± 0.11	1.20 ± 0.28
	Light trap	1988	64	0.52 ± 0.19	0.45 ± 0.15	1.44 ± 0.27
	Sunflowers	1986	8	0.43 ± 0.15	0.33 ± 0.11	1.17 ± 0.20
II.	Samples of moths collected with 'A' and 'B' attractants at other localities					
A.	Saskatoon, SK	1987	10	1.73 ± 0.13	0.28 ± 0.07	0.13 ± 0.04
	Stoughton, SK	1987	28	1.48 ± 0.26	0.27 ± 0.09	0.13 ± 0.07
	Edmonton, AB	1987	39	1.35 ± 0.34	0.21 ± 0.09	0.13 ± 0.07
	Fairview, AB	1987	8	1.16 ± 0.22	0.17 ± 0.06	0.05 ± 0.04
B.	Saskatoon, SK	1987	10	0.41 ± 0.09	0.27 ± 0.04	1.05 ± 0.17
	Stoughton, SK	1987	9	0.40 ± 0.17	0.39 ± 0.09	1.16 ± 0.43
	Edmonton, AB	1987	7	0.31 ± 0.06	0.26 ± 0.06	0.85 ± 0.19
III.	Moths collected at Ottawa with light trap, 1986 and 1987					
C.	Ottawa, ON		21	0.18 ± 0.06	0.83 ± 0.25	0.17 ± 0.07
D.	Ottawa, ON		13	1.20 ± 0.37	0.64 ± 0.13	0.63 ± 0.18

Neither of the sex attractants was effective at Ottawa, Ontario, Urbana, Illinois, Laramie, Wyoming, or Albuquerque, New Mexico, although at the Ottawa and Laramie locations many *F. jaculifera* were caught during the same period of time in light traps. No light traps were operated at the other locations.

EAD determinations of the antennal responses of moths caught in light

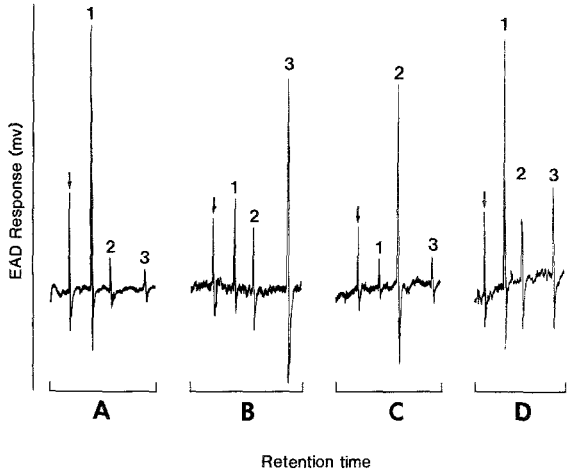


FIG. 3. GC-EAD responses of the four known pheromone types of *F. jaculifera* to a test blend containing equal concentrations of Z7–12 : Ac, Z9–14 : Ac, and Z11–16 : Ac. The vertical arrows indicate a nonspecific response to the hexane solvent. Peaks 1, 2, and 3 are the responses to Z7–12 : Ac, Z9–14 : Ac, and Z11–16 : Ac.

traps at Ottawa revealed two other pheromone types designated types C and D (Figure 3). Type C exhibited a strong response to Z9–14 : Ac and was obviously distinct from the two western types. Type D had a strong response to Z7–12 : Ac, similar to type A, but the responses to the other two components were significantly stronger (Figure 3, Table 1). Traps baited with the sex attractant for type A did not attract type D males at Ottawa and traps baited with reared D females did not attract A males at Lethbridge. Multivariate analyses of variance showed that the antennal responses of the four types represented discrete populations.

Pinned specimens of moths of the four pheromone types could not be distinguished by their appearance and no genitalic differences were found. Large series of specimens of types A and B both showed the wide range of dark to light phenotypes characteristic of the nominal species.

In the laboratory, the duration of development of type A is two to three weeks longer than that of type B, and in the field moths of A appear about two weeks later than those of B (Figure 4). However, there is considerable overlap of the flight periods, and, in view of the tendency for the females to mate a number of times, it is unlikely that this difference in phenology could maintain reproductive isolation in the absence of the sex pheromone differences.

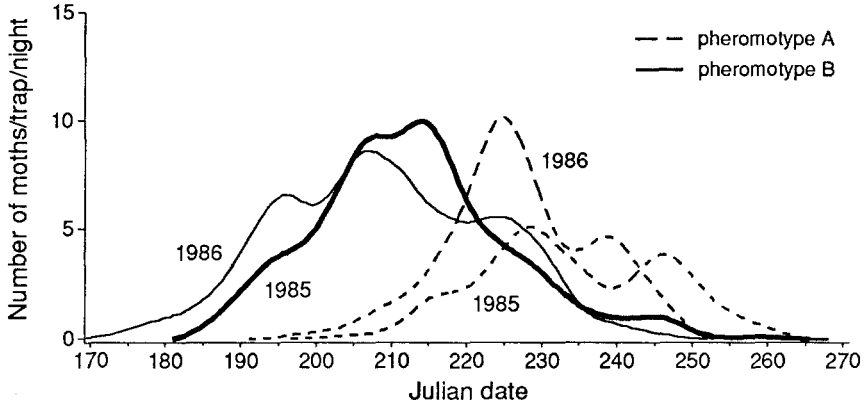


FIG. 4. Flight periods of *F. jaculifera* types A and B at Lethbridge, Alberta, in 1985 and 1986 based on catches of male moths in traps baited with the respective sex attractant.

DISCUSSION

Intraspecific differentiation of the sex pheromone system of a nominal species into pheromonal phenotypes was first documented in the European corn borer, *Ostrinia nubilalis* (Hbn.) (Kochansky et al., 1975) and has since been reported in several other species including the larch bud moth, *Zeiraphera diniana* Gn. (Guerin et al., 1984; Priesner and Baltensweiler, 1987a), the western avocado leafroller, *Amorbia cuneana* (Wlsm.) (Bailey et al., 1986), and the turnip moth, *Agrotis segetum* (Shiff.) (Lofstedt et al., 1986).

The European corn borer has two pheromonal phenotypes, which occur in both North America and Europe (Pena et al., 1988, and references therein). The Z-type has a 97:3 blend of *Z*11-14:Ac and *E*11-14:Ac and the *E*-type has the inverse blend. Males of both types give a similar pattern of electroantennographic response to blends of the two isomers when entire antennae are used as detectors, and they could not be distinguished by the GC-EAD method used in this study. However, males of the two types can be readily distinguished by the responses of individual olfactory sensilla (Hansson et al., 1987; Roelofs et al., 1987). In areas where the *Z*- and *E*-types occur sympatrically, hybridization is indicated by the presence of intermediate phenotypes (Klun and Cooperators, 1975; Anglade et al., 1984; Roelofs et al., 1985).

The two discrete pheromonal phenotypes of the larch bud moth utilize $>100:1$ and $1:>100$ blends of *E*9-12:Ac and *E*11-14:Ac, respectively (Priesner and Baltensweiler, 1987a). Although there is some correlation between host race and larval color form, the relationships are apparently dynamic, and intermediate phenotypes, presumably arising from hybridization and introgres-

sion, are present in some populations (Priesner and Baltensweiler, 1987a, b; Baltensweiler and Priesner, 1988).

In the western avocado leafroller three pheromonal phenotypes have been reported that utilize *E*, *Z*10, 12–14 : Ac and *E*, *E*10, 12–14 : Ac in ratios of about 8 : 1, 7 : 5, and 4 : 7 (Bailey et al., 1986). However, the relationship between the types, two of which are sympatric, has not yet been determined (Bailey et al., 1988).

European populations of the turnip moth, *A. segetum*, exhibit variation in sex pheromone (dialects) that, similar to the pheromone types of the dingy cutworm, involves shifts in the ratios of three pheromone components, i.e., *Z*5–10 : Ac, *Z*7–12 : Ac, and *Z*9–14 : Ac (Lofstedt et al., 1986). However, there are no reports of different dialects of the turnip moth occurring sympatrically. Within some populations of turnip moth the sex pheromone channel is quite broad; the relative proportions of the pheromone components vary considerably among females, and males are attracted to a wide range of blend ratios (Lofstedt et al., 1985). We have not determined the sex pheromone composition of individual dingy cutworm female moths, but males respond optimally to a relatively narrow range of component ratios (Byers and Struble, 1990).

Under natural conditions, reproductive isolation between the two pheromone types of the dingy cutworm that occur sympatrically on the northern Great Plains appears to be complete, and this also is likely to be true for the two pheromone types that occur sympatrically in central Canada. Male moths of sympatric types have antennal responses that are clearly of one type or the other, and intensive study of the two western types has shown no instance of cross-attractancy to either females or synthetic attractants. They are reproductively isolated and can be regarded as sibling species. The allopatric A and D types have the same main component, *Z*7–12 : Ac, and might represent populations along a cline of the amounts of the minor components relative to the main component. If so, the differentiation along the cline has been sufficient such that the sex pheromones of the two populations studied are not cross-attractive.

A communication system requires the simultaneous presence of two capabilities: signal production and signal reception. Sexual communication by means of chemicals probably arose early in the evolution of the Arthropoda (Kittredge and Takahashi, 1972). Subsequent differentiation to produce the present profusion and diversity of sex pheromones may then have proceeded via assortative mating of variants. A new pheromonal phenotype could evolve either according to "the genetic coupling hypothesis" whereby pheromone production and reception are controlled by pleiotropic or linked coadapted genes, or according to "the coevolution hypothesis" whereby selection acts to coordinate compatible but genetically independent pheromone production and reception mechanisms (Lofstedt et al., 1989). A new phenotype would seem most likely to evolve if the genes determining the male and female components of the system

were closely linked. However, in the European corn borer, the only lepidopteran species in which the genetics of the pheromone system have been studied in detail (Roelofs et al., 1987; Hansson et al., 1987), it has been shown that the genes determining variation in pheromone production by females and pheromone reception by males are not closely linked and are likely inherited independently (Lofstedt et al., 1989). Nevertheless, there is evidence of positive assortative mating in this species (Klun and Huettel, 1988).

In some species the pheromone system exhibits a high degree of constancy among geographic populations. In the cabbage looper, *Trichoplusia ni*, females from three populations across the United States produce similar quantities and blend ratios of the six known pheromone components (Haynes and Hunt, 1990). In the pink bollworm, *Pectinophora gossypiella* (Saund.), the blend ratio of the two pheromone components is similar throughout the worldwide range of this species (Haynes and Baker, 1988). Males of this species respond to a wide range of blend ratios, which would hinder the differentiation of populations using different blend ratios (Haynes and Baker, 1988). In *A. segetum* there is variation in the blend ratios produced by the females, which evidently has led to differentiation of allopatric dialects (Lofstedt et al., 1985). However, because of the broad range of response of the males, it is unlikely that the differentiation could be maintained in sympatry. In contrast, the response range of males of the dingy cutworm pheromone types to the attraction components of their females is narrower and differentiation of the pheromone communication channel, and its subsequent maintenance in sympatry, would seem more likely. Furthermore, some of the other components in the sex pheromone of the dingy cutworm appear to inhibit attraction between pheromone types and may play an important role in enhancing specificity.

The dingy cutworm is univoltine throughout a wide latitudinal range, and populations have undoubtedly undergone phenological adaptation to regional environments. Differentiation of the populations probably arose via assortative mating based on variations in the sex pheromone system. Selection for reinforcement of assortative mating to preserve coadapted gene complexes within populations of the dingy cutworm could then have resulted in differentiation of the pheromone types. We suspect that *F. jaculifera* consists of a mosaic of pheromonal phenotypes that are reproductively isolated and behave as sibling species wherever they occur in sympatry.

Note Added in Proof—Coexistence of two pheromone types of the turnip moth has been recently reported (Hansson, B.S., Toth, M., Lofstedt, C., Szocs, G., Subchev, M., and Lofqvist, J. 1990. Pheromone variation among eastern European and a western Asian population of the turnip moth, *Agrotis segetum*. *J. Chem. Ecol.* 16:1611–1622.

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IDENTIFICATION OF SEX PHEROMONES OF TWO SIBLING SPECIES IN DINGY CUTWORM COMPLEX, *Feltia jaculifera* (GN.) (LEPIDOPTERA: NOCTUIDAE)[1]

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Abstract—The sex pheromone components of the two sibling species of the dingy cutworm that occur on the prairies of western Canada were identified in abdomen-tip extracts from calling female moths. Three monounsaturated acetates, (Z)-7-dodecenyl acetate, (Z)-9-tetradecenyl acetate, and (Z)-11-hexadecenyl acetate, are common to both species in ratios of 100:13:3 for species A and 0.3:0.5:100 for species B. The most effective synthetic blends for the attraction of male moths in the field consisted of these three components in ratios of 10:1:0 at 8.8 μg/lure for species A and 1:1:2000 at 500 μg/lure for species B. The addition of Z5-12:Ac to either blend reduced the catches and the addition of Z7-12:OH or Z11-16:OH to the three-component blend reduced the catches of species B males. The species are morphologically indistinguishable, but the identity of the males attracted to the synthetic blends could be confirmed by their antennal responses to a test blend of the three components using a GC-EAD system. Both synthetic attractant blends are competitive with females and will be useful for studying the distribution, biology, and relative abundances of the two species.

Key Words—*Feltia jaculifera*, *Feltia ducens*, dingy cutworm, Lepidoptera, Noctuidae, sex pheromone, pheromone polymorphism, pheromonal phenotypes, sibling species, (Z)-7-dodecenyl acetate, (Z)-9-tetradecenyl acetate, (Z)-11-hexadecenyl acetate.

INTRODUCTION

Male moths of the dingy cutworm, *Feltia jaculifera* (Gn.), were first reported to be attracted to synthetic blends containing (Z)-11-hexadecenyl acetate

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(Underhill et al., 1977). Steck et al. (1982, 1986) identified Z11–16:Ac as the main sex pheromone component of the dingy cutworm and showed that a three-component blend of Z7–12:Ac, Z9–14:Ac, and Z11–16:Ac in a ratio of 1:1:1000 and at 20–100 μg/septum dispenser was an effective lure. However, they were unable to detect either Z7–12:Ac or Z9–14:Ac in female abdomen tip extracts nor were they able to confirm the identification of Z11–16:Ac by a complete mass spectrum (Steck et al., 1986).

From 1975 to 1982, we also were studying the attraction of dingy cutworm male moths to various multicomponent blends and found that moths of this species were consistently attracted during July and August to Z11–16:Ac alone or in blends with ca. 1% of Z7–12:Ac and Z9–14:Ac. However, from mid-August until the end of the flight, males of what appeared to be the same species were attracted by various two-component blends of Z7–12:Ac and Z9–14:Ac, with Z7–12:Ac as the main component. In 1985, we discovered by determining the antennal responses of male moths collected throughout the flight period that two distinct pheromone types were present (Byers et al., 1990).

In this paper, we report the identification of the sex pheromone components of both pheromone types, hereafter referred to as species A and species B. Z7–12:Ac is the main component of the sex pheromone of species A and, although small quantities of both Z9–14:Ac and Z11–16:Ac are present in female extracts, only Z9–14:Ac is required for the two-component attractant specific for this species. The three-component reported by Steck et al. (1986) is specific for species B. We confirm that Z11–16:Ac is the major sex pheromone component of this species and demonstrate the presence of Z7–12:Ac and Z9–14:Ac in female extracts.

METHODS AND MATERIALS

The moths used in this study were from isofemale lines originating from moths collected at Lethbridge, Alta. Larvae were reared on an artificial diet (Hinks and Byers, 1976).

Pheromone was recovered from calling 4- to 10-day old females kept at 17 ± 2°C, ca. 60% relative humidity and 16:8 light–dark photoperiod. Calling behavior of both species peaked between 3 and 5 hr into the scotophase. The everted ovipositors were removed with fine forceps and soaked for about 48 hr in *n*-hexane after removal of adherent abdominal tissue. Extracts were filtered and reduced to 0.5 female equivalents (FE)/μl under N_2 without further cleanup. Most extracts contained material from about 50 abdomen tips. During the study, 750 female moths were used. Separate extracts were obtained from three isofemale lines of species A and from two isofemale lines of species B.

Gas chromatography (GC) analyses were done with a Hewlett-Packard

model 5830 using a flame ionization detector (FID), and with a modified Varian model 2700 with simultaneous use of a FID and an electroantennographic detector (EAD) (Struble and Arn, 1984). The effluent split ratio between the FID and EAD detectors was 2 : 1. Capillary columns used for the GC analyses were borosilicate with Carbowax 20 M TPA (20 and 43 m × 0.32 mm ID with film thickness 0.25 μm) prepared by the dynamic method of Grob et al. (1977) and fused silica with Supelcowax 10 (30 m × 0.32 mm ID with film thickness 0.25 μm). Helium carrier gas linear velocity was 30 cm/sec (at 50°C) for all analyses, and various temperature programs were used with splitless injections.

Mass spectra were obtained with a Hewlett-Packard model 5985B GC-mass spectrometer (GC-MS) using chemical ionization (CI) with isobutane as the reactant gas. Borosilicate capillary columns were used with Carbowax 20 M TPA (28 m × 0.32 mm ID with film thickness 0.24 μm) and SP 2340 (75 m × 0.32 mm ID with film thickness 0.06 μm). Fused silica columns were used with Supelcowax 10 (30 m × 0.32 mm ID with film thickness 0.25 μm) and FFAP (25 m × 0.25 mm ID with film thickness 0.25 μm). A typical temperature program was 50°C, 1.0 min, 50 to 125°C at 30°C/min, and 3°C/min, to 190°C.

Synthetic pheromone components were either synthesized or purchased. The acetates were purified by argentation liquid chromatography (Houx et al., 1974) to chemical purity >99% and isomeric purity >99.8%. Alcohols of similar purity were obtained by treatment of the corresponding acetate with methanolic potassium hydroxide. Synthetic blends were prepared in *n*-hexane. Red rubber septa (Arthur H. Thomas Co. Catalog No. 1780-J07) were used as dispensers and traps were of the cone-orifice type (Struble, 1983). Treatments were replicated four times in a randomized block design within a single row in a north–south direction along the edge of cereal crops. Within the row, traps were positioned 20 m apart at a height of 1 m. A guard trap baited with the best attractant known at the time of the test was placed at each end of the row to reduce the end effect. Catches in the guard traps were not included in the experimental data. The traps were emptied and catches recorded at least twice a week. Analysis of variance was done on transformed ($\sqrt{x + 1}$) data, and treatment means were compared by Duncan's multiple range test. In all tables, mean catches of moths within a test (column) followed by the same letter do not differ ($P > 0.05$).

The attractancy of optimized blends was compared with that of unmated females in field tests. Reared female moths of known species were confined in plastic mesh cages 5 cm in depth and of a diameter (ca. 10 cm) such that they would fit snugly within the body of the trap. Each cage was provided with a vial containing water and a dental roll wick. Preliminary experiments indicated that the presence of a cage reduced trap catches; therefore the synthetic lures were also placed within cages. Traps baited with four virgin female moths each

or the attractant blend were positioned 10 m apart in pairs with four replicates at least 200 m apart. To reduce mortality resulting from high daytime temperatures, the bait females were removed from the field from 0800 to 1630 hr.

All captured moths were identified by the authors, with confirmation of representative specimens by J.D. Lafontaine (Biosystematics Research Centre, Agriculture Canada, Ottawa, Ontario). Identification of representative specimens of the two species was established by their antennal responses (GC-EAD) to a test blend of Z7–12 : Ac, Z9–14 : Ac, and Z11–16 : Ac in a ratio of 1 : 1 : 1 as described by Byers et al. (1990).

RESULTS AND DISCUSSION

GC and GC-MS Analyses. Typical reconstructed chemical ionization (CI, isobutane), total-ion chromatograms of abdomen-tip extracts of female moths of species A and B are illustrated in Figure 1. The corresponding mass chromatograms for the major pheromone components based on M + 1 ions for monounsaturated acetates (227, C_{12}; 255, C_{14}; and 283, C_{16}) are also illustrated. These data clearly show that the major pheromone components of species A and B are Z7–12 : Ac and Z11–16 : Ac, respectively.

Complete CI differential mass spectra, which were consistent with those of authentic chemicals, were obtained for the pheromone components listed in Table 1. The total-ion chromatogram data for both species were scanned for other C_{10}–C_{16} saturated, mono-, and diunsaturated aldehydes, alcohols, and acetates, but none were detected. The components detected by GC-MS all cochromatographed with authentic samples. However, Z5–16 : Ac and Z7–16 : Ac were not completely resolved by any of the columns used, so the Z7–16 : Ac indicated by GC-MS in species B might include some Z5–16 : Ac. Most of the other positional and geometrical isomers of monounsaturated C_{10}–C_{16} acetates and alcohols with the double bond beyond C_4 were clearly resolved. The isomer assignments of the pheromone components of both species were consistent with the results of GC-EAD analyses and field-attractancy tests.

EAD-FID Analyses. EAD-FID chromatograms of female abdomen-tip extracts of both species are illustrated in Figure 2. Antennae of species A males gave consistent responses to Z5–12 : Ac, Z7–12 : Ac, and Z9–14 : Ac in extracts from species A females and antennae of species B males consistently responded to Z7–12 : Ac, Z9–14 : Ac, Z11–16 : Ac, and Z11–16 : OH in extracts from species B females. Comparable responses were obtained with the corresponding synthetic chemicals (Table 1), corroborating the structural assignments of these components.

The isomer assignment of some of the pheromone components in the extracts was verified by using, as the GC-EAD detector, the antennae from a

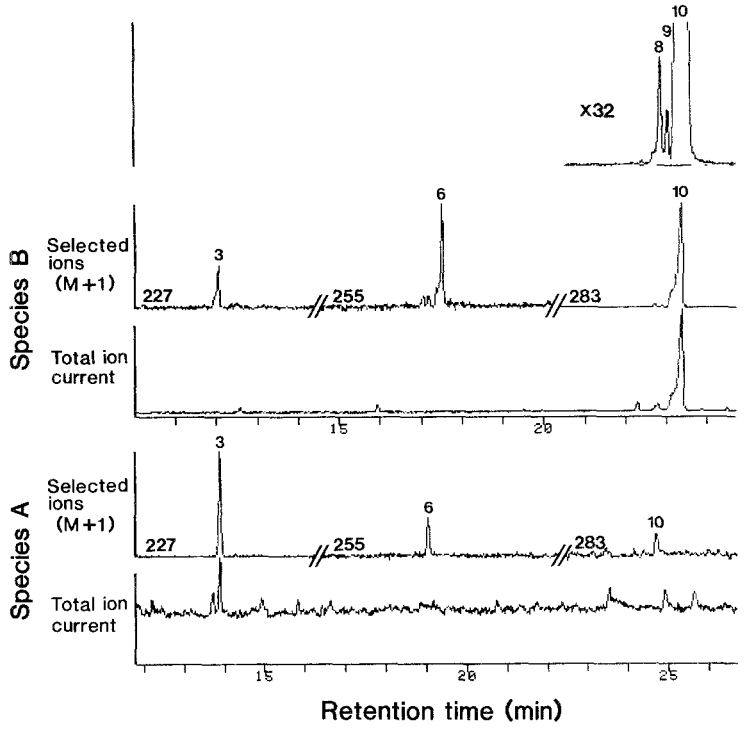


FIG. 1. Total ion chromatogram, CI, and selected ion mass chromatogram of the pheromone components of abdomen-tip extracts of females of species A (bottom) and B (top). The retention times for the two extracts do not match because different columns were used. The components are: Z7–12:Ac (peak 3), Z9–14:Ac (6), Z7–16:Ac (8), Z9–16:Ac (9), and Z11–16:Ac (10). For species B, the M+1 ion 283 is also shown at 32 × amplification.

male moth of another species known to give a strong response to a specific component (Struble and Arn, 1984). Antennae of *Euxoa ochrogaster* (Gn.) are moderately sensitive to Z7–12:Ac (Struble et al., 1980) and confirmed the presence of this compound in both species A and B. *E. ochrogaster* antennae are also very sensitive to Z5–12:Ac and confirmed the presence of this component in species A and indicated its presence in species B at a level not detectable by GC-MS. Antennae of *Lacinipolia stricta* (Wlk.) males are very sensitive to Z9–14:Ac and moderately sensitive to Z7–12:Ac (unpublished results) and confirmed the presence of these components in extracts of both species. Antennae of *Euxoa messoria* (Harr.) males are moderately sensitive to Z11–16:Ac (Struble and Byers, 1987) and were used to confirm the presence of this component in both species.

TABLE 1. PHEROMONE COMPONENTS PRESENT IN ABDOMEN-TIP EXTRACTS OF FEMALES AND ANTENNAL SENSITIVITY OF MALES

Component	Species A			Species B		
	Female extract			Female extract		
	GC-MS	Relative amount[a]	Male antenna Sensitivity[b]	GC-MS	Relative amount[a]	Male antenna Sensitivity[b]
1. 12:Ac	+[c]	9.8	0.40	+	0.6	0.32
2. Z5-12:Ac	+	0.6	0.50	ND		0.36
3. Z7-12:Ac	+	100	1.63	+	0.3	0.65
4. Z7-12:OH	+	49.8	0.00	ND		0.12
5. 14:Ac	+	1.8	0.12	+	0.8	0.38
6. Z9-14:Ac	+	13.2	0.31	+	0.5	0.53
7. 16:Ac	+	0.8	0.00	+	5.7	0.35
8. Z7-16:Ac	ND		0.05	+[d]	1.6	0.58
9. Z9-16:Ac	ND		0.09	+	1.0	0.34
10. Z11-16:Ac	+	2.6	0.11	+	100	1.10
11. Z11-16:OH	ND		0.11	+	3.6	0.18
ng/FE (main component)		0.3–0.5			10–20	

[a] Based on GC-MS analyses of hexane extracts of female abdomen tips.
[b] Typical response (mV) of male antennae to 1.3 ng of synthetic injected splitless on a Supelcowax 10 fused silica column (30 m × 0.32 mm ID) at 50°C for 0.2 min, 25°C/min to 110°C, 4°C/min to 205°C; the EAD-FID split ratio was 2:1.
[c] +, Complete spectra obtained; ND, not detectable.
[d] Probably Z7-16:Ac but resolution of Z5/Z7-16:Ac incomplete.

Species A Field Attractancy Tests. Males of species A were consistently attracted to blends of Z7-12:Ac and Z9-14:Ac with a 10:1 ratio being most attractive (Table 2). This is essentially the same ratio of these components present in the female extracts. The identification of representative samples of captures moths by their antennal responses (GC-EAD) confirmed that all the moths attracted to the two-component blends in this experiment were of species A.

To determine the effect of the other monounsaturated components detected in the female extracts, additional components were added singly or in combinations to a 500 ng and 50 ng/septum blend of Z7-12:Ac and Z9-14:Ac. Field tests were done with Z5-12:Ac (50 ng), Z7-12:OH (200 ng), and Z11-16:Ac (25 and 100 ng), which were present in the extracts of this species, as well as the following geometrical and positional isomers, E7-12:Ac (50 ng), Z7-14:Ac (25 and 100 ng), and Z11-14:Ac (5, 25, and 100 ng). Z5-10:Ac (5 ng), Z7-10:Ac (5 ng), and Z9-14:OH (25 ng) also were tested as third components. These components were tested in a total of 38 treatments in four rep-

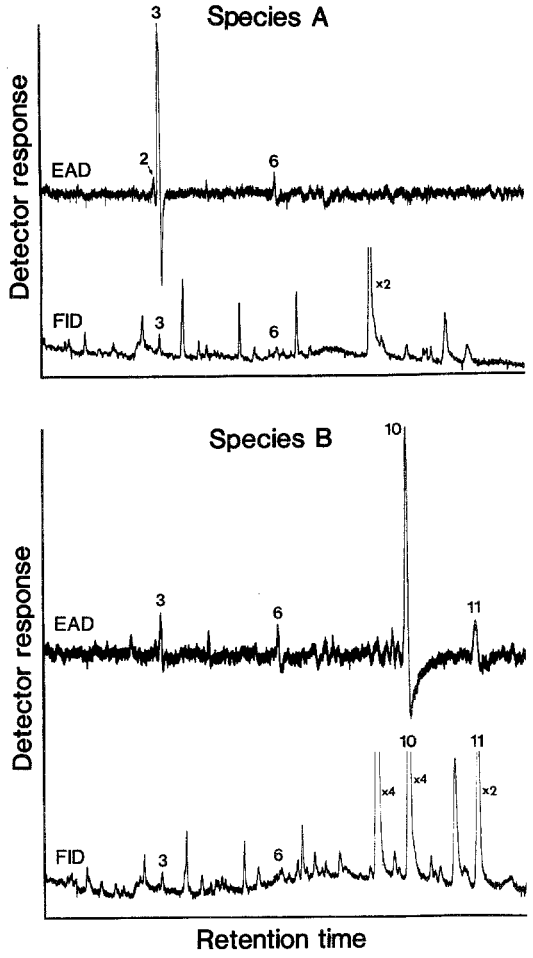


FIG. 2. Gas chromatograms with FID and EAD responses to 1.0 female equivalent (splitless injection) of abdomen-tip extracts of females of species A (top) and B (bottom), on a 30 m × 0.32 mm ID Supelcowax 10 fused silica column. The EAD responses are to Z5–12:Ac (peak 2), Z7–12:Ac (3), Z9–14:Ac (6), Z11–16:Ac (10), and Z11–16:OH (11).

lications. A total of 10,420 male moths were captured in these tests, but none of the additional components improved attractancy ($P > 0.05$). The presence of Z5–10:Ac or Z5–12:Ac as the third component reduced attractancy. The blend containing Z5–12:Ac also attracted pale western cutworm males as would be expected (Struble and Swailes, 1978).

TABLE 2. NUMBERS OF MALES OF SPECIES A CAUGHT IN TRAPS BAITED WITH LURES CONTAINING Z7-12:Ac (500 ng) PLUS VARIOUS AMOUNTS OF Z9-14:Ac

Z7-12:Ac (ng/lure)	Z9-14:Ac (ng/lure)	Mean No. males/trap[a]
500	200	65.5c
500	100	93.3ab
500	50	112.5a
500	25	84.0bc
500	10	72.3bc
500	1	44.0d

[a] Four replications, August 22–30, 1983. Traps baited with the three-component blend for species B (Z7-12:Ac, Z9-14:Ac, and Z11-16:Ac at 0.1, 0.1, and 200 μg/dispenser) caught 27.8 species B males/trap during this test.

The attractancy of the two-component blend, Z7-12:Ac and Z9-14:Ac at 2.0 and 0.2 μg/septum, was compared with that of virgin females in the field from August 22 to 28 1987. The mean numbers of males of species A attracted by the females and the synthetic blend were 53 and 71/trap, respectively. Antennal responses were determined for 69 of the males caught at the females, and all were species A (see Figure 3c in Byers et al., 1990). Males of nontarget species were <2% of the moth catch at both the synthetic lures and females.

The above results show that the two-component blend of Z7-12:Ac and Z9-14:Ac at a ratio of 10:1 is an effective and specific attractant for species A of the dingy cutworm. To establish the quantity per lure that would function best throughout an average flight period of four to six weeks, this two-component blend was tested at seven dispenser loads (Table 3). It was concluded from the data obtained (Table 3, Figure 3) that the 8.8 μg/septum load would be the most useful for monitoring purposes.

Species B Field Attractancy Tests. Preliminary tests indicated that species B males were attracted to blends containing Z11-16:Ac as the main component in combination with relatively small amounts of Z7-12:Ac and Z9-14:Ac. Subsequent field attractancy tests with numerous different ratios of these components established that the blend of Z7-12:Ac, Z9-14:Ac and Z11-16:Ac at 0.1, 0.1 and 200 μm/septum was an effective attractant for species B. This is similar to the three-component blend of Z7-12:Ac, Z9-14:Ac, and Z11-16:Ac at 0.1, 0.1, and 100 μg/septum reported by Steck et al. (1986) as an effective attractant for the dingy cutworm. Determination of the antennal responses of captured moths by GC-EAD showed that both blends were specific for species B.

To determine the effect of the other monounsaturated components detected

TABLE 3. MEAN NUMBERS OF SPECIES A MALES ATTRACTED TO BLEND OF Z7-12:Ac AND Z9-14:Ac IN A RATIO OF 10:1 AT VARIOUS LOADS PER SEPTUM

Quantity (μg/septum)	Mean males attracted[a]		
	Aug. 8-22	Aug. 23-Sept. 17	Total catch Aug. 8-Sept. 17
0.14	30.0c	2.0e	32.0cd
0.55	61.7b	10.2d	71.9bc
2.2	93.3a	32.5c	125.8ab
8.8	80.2ab	66.5a	146.7a
35.2	41.5bc	69.7a	111.2ab
140.8	8.8d	36.7bc	45.5c
563.2	1.5d	15.3d	16.8d

[a] Four replications.

in species B female extracts on the attraction of males, additional components were added singly or in combinations to a 0.1, 0.1, and 200 μg/septum blend of Z7-12:Ac, Z9-14:Ac, and Z11-16:Ac. Field tests were done with Z5-12:Ac (0.1 μg), Z7-16:Ac (5 μg), Z9-16:Ac (3 μg), and Z11-16:OH (1.0 and 10 μg), which had all been detected in the extracts, although the presence of Z5-12:Ac was indicated only by the EAD response of *E. ochrogaster* antennae. In addition, Z5-10:Ac (0.05 μg), Z7-12:OH (0.1 μg), Z9-12:Ac (0.1

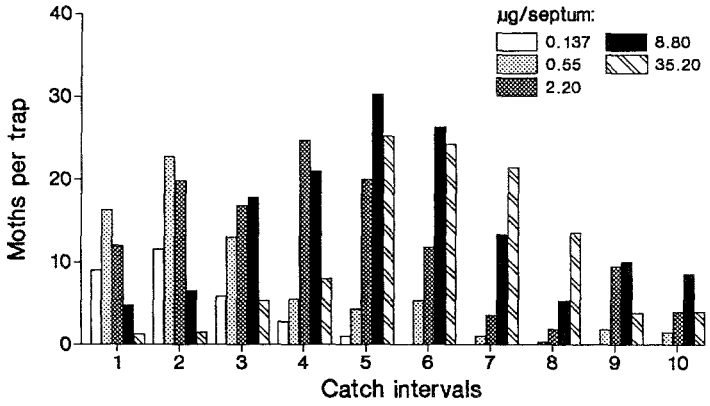


FIG. 3. Numbers of male moths of species A, caught at three-day intervals with lures of Z7-12:Ac and Z9-14:Ac (10:1) at various loads per septum, August 8-September 6, 1984, four replications.

μg), Z7–14:Ac (0.1 μg), Z9–14:OH (0.1 μg), and Z11–14:Ac (0.1 μg) were also tested as fourth components. These components were tested in a total of 21 treatments in four replications. A total of 4673 moths were captured in these tests. The presence of Z5–12:Ac (0.1 μg), Z7–12:OH (0.1 μg), or Z11–16:OH (10 μg) as a fourth component reduced the catches of males, but none of the other components or Z11–16:OH at 1.0 μg, had any apparent effect ($P > 0.05$).

The attractancy of the three-component blend of Z7–12:Ac, Z9–14:Ac, and Z11–16:Ac at 0.1, 0.1, and 200 μg/septum was compared with that of virgin females in two separate tests. In a test from July 30, to August 8, 1985, the traps baited with females caught 53 males/trap and those baited with lures caught 45 males/trap. In another test from July 29, to August 4, 1987, the catches at female or lure-baited traps were 29 and 24 males/trap, respectively. The ratio of males caught at the females versus the synthetic was similar in both tests. In 1985, the antennal responses of 40 of the males caught at the females were determined by GC-EAD, and all were species B. In the 1987 test, 68 males caught at the females were checked, and all were species B (see Figure 3c in Byers et al., 1990). The three-component blend attracted few ($< 1\%$) males of nontarget species, but the traps baited with females caught 54 dark-sided cutworm and 10 clover cutworm male moths during the above tests. Both these species have Z11–16:Ac as their main pheromone component. The essential secondary component for attraction of the dark-sided cutworm is Z7–16:Ac (Struble and Byers, 1987) and Z11–16:OH for attraction of clover cutworm (Struble and Swailes, 1977). Both these components were present in the female extracts (Table 1) and were evidently also present in the sex pheromone released by the females.

The 0.1:0.1:200 blend of Z7–12:Ac, Z9–14:Ac, and Z11–16:Ac was tested at dispenser loads of 50, 200, 500, and 1000 μg/septum to determine which quantity would be best for monitoring over a period of four to six weeks. Dispensers with loads of 200 μg/septum that had been aged in the field for two or four weeks also were included in the test. During the 30-day duration of the test, the 500-μg treatment caught the most moths, 127.5/trap, although the catches were not significantly greater than those with the fresh 200 μg treatment. The 500 μg/septum load would probably be the most suitable for monitoring.

The width of the sex pheromone channels of the two sibling species was investigated using intermediate blends made by adding various amounts of Z11–16:Ac to the optimized blend for species A (Table 4). When the amount of Z11–16:Ac was 2 μg or less, the males caught were nearly all species A. As the amount of Z11–16:Ac was increased from 4 to 50 μg, the catches shifted

TABLE 4. CATCHES OF SPECIES A AND B MALES AT INTERMEDIATE AND STANDARD BLENDS

Blends (μg/septum)			No. of males caught	Species determined by GC-EAD		
Z7-12 : Ac	Z9-14 : Ac	Z11-16 : Ac		No. checked	No. species A	No. species B
Exp. 1[a]						
2	0.2	0*[b]	1,656	56	56	0
2	0.2	1	1,324	84	83	1
2	0.2	10	281	50	22	28
2	0.2	50	268	47	7	40
2	0.2	100	232	42	1	41
2	0.2	200	221	44	0	44
0.1	0.1	200**[b]	289	47	0	47
Exp. 2						
2	0.2	0*	180	0		
2	0.2	1	110	10	10	0
2	0.2	2	111	14	14	0
2	0.2	4	74	18	14	4
2	0.2	6	77	8	1	7
2	0.2	8	68	11	1	10
2	0.2	10	86	11	3	8
0.1	0.1	200**	301	0		

[a] Experiment 1 conducted August 2–September 3, 1985; lures renewed August 21. Experiment 2 conducted August 9–23, 1985.
[b] *, **Standard blends for species A and B, respectively.

from being mostly species A to being mostly species B. When the amount of Z11-16 : Ac was 100 μg or more, the males caught were nearly all species B. The blend ratios that specifically attract one species or the other are relatively narrow but both species are attracted to a wide range of intermediate ratios.

The two-component blend of Z7-12 : Ac and Z9-14 : Ac at a ratio of 10 : 1 and a load of 8.8 μg/septum is an effective attractant for males of species A for four to six weeks, while the three-component blend of Z7-12 : Ac, Z9-14 : Ac, and Z11-16 : Ac at 1 : 1 : 2000 and 500 μg/septum is an effective attractant for species B for a similar period. These blends will be useful for determining the distribution, ecology, and relative abundances of these two sibling species of the dingy cutworm.

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NEOTROPICAL ANT GARDENS II. BIOASSAYS OF SEED COMPOUNDS

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Abstract—A number of volatile compounds occur on the seeds of taxonomically unrelated ant-garden epiphytes in western Amazonia. In field trials in southeastern Peru, we assayed the responses of ant-garden ants (*Camponotus femoratus*) to these and structurally similar compounds applied to artificial "seeds" made from zeolite molecular sieves. Benzothiazole, **2,** present on seeds of eight ant-garden epiphytes, repelled ants over the range of concentrations tested, as did 1-(2-hydroxy-6-methylphenyl)ethanone, **3,** occurring on seeds of six ant-garden epiphytes. 2-Hydroxy-6-methylbenzoic acid, methyl ester (methyl-6-methylsalicylate, 6-MMS), **1,** found on seeds of at least nine ant-garden epiphytes, was mildly repellent at high concentration, but stimulated excitement, seed handling, and (rarely) seed carrying at lower concentrations. Vanillin, **5,** a seed compound of four ant-garden epiphytes, and limonene, **6,** a monoterpene from seeds of three ant-garden epiphytes, both stimulated excitement, alarm, seed handling, and (rarely) seed carrying. Identified from seeds of seven ant-garden epiphytes, 1-(2,4-dihydroxyphenyl)ethanone, **4,** elicited little or no response. Among 70 compounds tested (mainly aromatic compounds), those found on seeds of ant-garden epiphytes or having structural features in common with such compounds were the most attractive to the ants. Although not present on epiphyte seeds, 2-hydroxy-3-methoxybenzenemethanol, **10,** consistently stimulated seed transport to the nest in one year, but did so only rarely in subsequent years. Some of the volatile compounds on seeds of ant-garden epiphytes probably play a role in

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ant attraction to epiphyte seeds, but evidence remains ambiguous. Finally, *Ca. femoratus* responded to one test compound [1-(2-hydroxy-4-methoxyphenyl)ethanone, **60**] (absent from epiphyte seeds) by descending from the vegetation to the ground.

Key Words—Ant, ant-garden, benzothiazole, *Camponotus*, Hymenoptera, Formicidae, chemical mimicry, epiphyte, 2-hydroxy-3-methoxybenzenemethanol, 1-(2-hydroxy-4-methoxyphenyl)ethanone, 2-hydroxy-6-methylbenzoic acid, methyl ester, 1-(2-hydroxy-6-methylphenyl)ethanone, 1-(2,4-dihydroxyphenyl)ethanone, limonene, 6-methyl-methylsalicylate, 6-MMS, seed dispersal, symbiosis, tropical forests, vanillin.

INTRODUCTION

Among symbiotic ant–plant relationships, perhaps none is so enigmatic as Neotropical ant gardens (Davidson, 1988; Davidson and Epstein, 1989; Seidel et al., 1990). Within a single locality, as many as 11 unrelated epiphyte species are cultivated together in gardens on the arboreal nests of two cohabiting ant species. *Camponotus femoratus* and *Crematogaster* cf. *limata parabiotica* cooperate in building shared nests of carton and organic detritus. Workers of the former species carry epiphyte seeds into brood chambers. Seeds survive unharmed to germinate, and plants grow to reproductive size in the rich organic medium of nest gardens. Workers of both ant species retrieve animal feces, which may fertilize their epiphytes, and at least *Ca. femoratus* protects its plants from insect herbivores (Davidson and Epstein, 1989). It is likely that all ant-garden epiphyte species depend on these beneficial services from ants in order to establish and grow to reproductive size, although some of these plants can continue to reproduce after the ants have deserted older nest sites (Davidson, 1988).

Seed dispersal by ants appears to be fundamental to the ant–garden symbiosis, but the basis of ant attraction to seeds is poorly understood at present (Davidson, 1988; Davidson and Epstein, 1989). Although seeds of some of these plants have nutritional appendages (arils or elaiosomes), those of other species bear only gelatinous fruit pulp. Moreover, in cafeteria experiments, ants ranked seeds of the various epiphyte species in a distinctive preference order that correlated poorly with both short-term and long-term expectation of resource gain from the plants. Workers of *Ca. femoratus* also accurately discriminated seeds of one ant-garden epiphyte from those of a congeneric species even after the seeds had passed through the digestive tracts of frugivorous bats. Together, these observations suggest that ants might be attracted to seeds by nonnutritional cues.

Recently, Seidel et al. (1990) identified volatile compounds common to the seeds of most ant-garden epiphytes. Table 1 lists these substances and gives

TABLE 1. REPRESENTATION OF SEED COMPOUNDS ACROSS EPIPHYTE SPECIES[a]

Seed compound	Number of epiphyte species
Aromatic compounds	
2-Hydroxy-6-methylbenzoic acid, methyl ester (**1**)	8M, 1T
Benzothiazole (**2**)	7M, 1T
1-(2-Hydroxy-6-methylphenyl)ethanone (**3**)	4M, 2T
1-(2,4-Dihydroxyphenyl)ethanone (**4**)	3M, 4T
4-Hydroxy-3-methoxybenzaldehyde (**5**)	3M, 1T
Monoterpenes	
Limonene (**6**)	3M
α-Pinene (**7**)	1M, 1T
β-Pinene (**8**)	1M, 1T
trans-Pinocarveol (**9**)	1M

[a] Compounds found in measurable (M) or trace (T) quantities (Seidel et al., 1990).

the number of ant-garden epiphyte species whose seeds have the compound as a measurable or trace component. To determine if seed volatiles were attractive to *Ca. femoratus*, we conducted field trials with these and structurally related compounds loaded onto artificial "seeds" made from zeolite molecular sieves. Ants often exhibit strong responses to various odorous substances, and we purposely included a range of chemicals to provide a background of ant responses against which responses to actual seed compounds could be evaluated. Here, we report the results of our bioassays as well as the synthetic methods for all test compounds whose syntheses were novel.

METHODS AND MATERIALS

Preparation and Selection of Chemicals for Bioassays

Rationale. Compounds used in bioassays occurred in either ant-garden epiphytes or ants, or were structurally related to such compounds. Different suites of compounds were field tested in different years. In 1984, before the identities of seed compounds had been determined, we examined ant responses to vanillin, **5**, and to 13 other structurally similar compounds (see Table 2 below). This decision was based on the vanillin-like aroma of epiphyte seeds and rare occurrences of *Vanilla planifolia* in ant gardens (Davidson, 1988). Limonene, **6**, also was tested because of the conjecture (later rejected, Seidel, 1988) that the essential oils of regular ant-garden host trees in the genus *Calyptranthes* included simple terpenes.

In later years, after seed compounds had been identified, field bioassays focused on 2-hydroxy-6-methylbenzoic acid, methyl ester (methyl 6-methylsalicylate, 6-MMS), **1,** and a variety of other benzoic acid esters, the related benzoic acids, **5** and its isomeric benzaldehydes, 1-(2-hydroxy-6-methylphenyl)ethanone, **4,** and its isomers and analogs, and benzothiazole, **2.** Also included among test compounds were limonene, **6** (see Table 1), and several other monoterpenes. The various benzenemethanols were selected because of the positive results with 2-hydroxy-3-methoxybenzenemethanol, **10,** during the 1984 field bioassays. Skatole, **70,** was included because of the tendency of ant-garden ants to collect animal feces and seeds from these feces (Davidson, 1988).

The remaining target compounds were selected for a variety of reasons (Seidel, 1988), but in general we sought to provide a variety of substitution patterns and functionality characteristic of compounds found on ant-garden epiphytes and ants, or related species. This was necessary to determine whether ant responses occurred principally to particular chemical structures or were broadly similar over a diversity of odorous compounds.

Quality Control. Once the selected compounds were obtained through purchase or synthesis, they were purified to 99.9% or greater purity by recrystallization or chromatography. Purity was checked by HPLC or GLC (Seidel, 1988).

Synthesis. The synthesis of most compounds was accomplished in a single step using the appropriate synthon. Details of these syntheses are recorded elsewhere (Seidel, 1988). All synthesis reagents obtained from commercial suppliers were used without further purification. IR spectra were obtained with a Perkin-Elmer 298, Perkin-Elmer 1500, or Bio-Rad (Digilab) FTS-40 spectrophotometer with absorbances reported in wavenumbers (cm^{-1}), and relative intensities reported as strong (s, 0–33% transmittance), medium (m, 34–66% transmittance), or weak (w, 67–100% transmittance). [^{1}H]NMR spectra were recorded on a Varian EM-390 or XL-300 spectrometer. Chemical shifts are given in parts per million (ppm) relative to tetramethylsilane (δ 0) as internal reference. Mass spectrometric (MS) determinations were carried out on a VG Analytical 7070E double-focusing mass spectrometer at 70 eV, unless noted, and are reported as *m/z* (relative intensity). Melting points were obtained on a Mel Temp melting point apparatus and are uncorrected. Thin-layer chromatography analyses were performed on precoated sheets (0.2 mm thick) of silica gel 60F (E. Merck) with detection by UV, phosphomolybdic acid, or 2,4-dinitrophenylhydrazine reagent. Flash chromatography took place on 230–400 mesh ASTM silica gel 60 (EM reagents). Medium-pressure liquid chromatography (MPLC) was performed on 230–400 mesh ASTM silica gel 60 (EM reagents) using a Milton Roy mini-pump and Altex columns. Ethyl acetate (EtOAc) and hexanes (Hex) were distilled prior to MPLC use and spectral grade solvents used for spectroscopic measurements. Tetrahydrofuran (THF), ether, and tol-

uene were freshly distilled from potassium-benzophenone ketal. Dichloromethane (CH_2Cl_2) was freshly distilled from calcium hydride. Methanol (MeOH) was distilled from magnesium and stored over 4 Å molecular sieves. Spectral-grade acetone was used without prior treatment. All reactions were run under nitrogen or argon atmospheres unless otherwise noted.

4-Hydroxy-3-methoxybenzoic Acid, Methyl Ester (**22**). Purification by MPLC on a 5 × 50-cm column using 40% (v/v) EtOAc–Hex gave **22** as a white solid: mp 64.5–65.0°C; lit 60–62°C (Rao *et al.*, 1981); R_f 0.17 (20% (v/v) EtOAc–Hex); IR (melt) 3600–2900 m, 1698 s, 760 s; [^{1}H]NMR ($CDCl_3$) 3.84 (s, 3H), 3.88 (s, 3H), 6.22 (br s, 1H), 6.92 (d, J = 9 Hz, 1H), 7.59 (m, 2H); MS 182 (60), 151 (100), 123 (7), 69 (9).

2-Hydroxybenzenemethanol (**25**). Purification by flash chromatography on a 1 × 25-cm column using 75% (v/v) EtOAc–Hex gave **25** as a white solid: mp 84–85°C; lit 85–86°C (Covert et al., 1932); R_f 0.31 (35% (v/v) EtOAc–Hex); IR (mineral oil) 3550–2500 s, 985 s, 735 s; [^{1}H]NMR ($CDCl_3$–acetone-d_6) 3.22 (br s, 2H), 4.73 (s, 2H), 6.60–7.22 (m, 4H); MS 124 (44), 106 (85), 78 (100), 77 (39), 69 (15), 51 (22), 39 (22).

2-Hydroxy-3-methylbenzenemethanol (**26**). Purification by flash chromatography on a 3 × 20-cm column using 20% (v/v) EtOAc–Hex gave **26** as a clear colorless oil: lit mp 32.8–33.8°C (Hanus, 1940); R_f 0.26 [35% (v/v) EtOAc–Hex]; IR (neat) 3650–2800 s, 1090 s; [^{1}H]NMR ($CDCl_3$) 2.21 (s, 3H), 2.90 (br s, 1H), 4.69 (d, 2H), 6.69 (d, J = 6 Hz, 1H), 6.73 (s, 1H), 7.00 (d, J = 6 Hz, 1H), 7.33 (br s, 1H); MS 138 (33), 121 (11), 120 (100), 92 (42), 91 (95), 77 (12), 65 (16), 51 (11), 39 (18).

2-Hydroxy-4-methylbenzenemethanol (**27**). Purification by flash chromatography using 20% (v/v) EtOAc–Hex gave **27** as a shiny, white solid: mp 107–107.5°C; Lit 103°C (Dunning, et al., 1936); R_f 0.18 [35% (v/v) EtOAc–Hex]; IR (mineral oil) 3550–2500 s, 1090 s; [^{1}H]NMR ($CDCl_3$–acetone-d_6) 2.23 (s, 3H), 4.09 (t, J = 4.5 Hz, 1H), 4.70 (d, J = 4.5 Hz, 1H), 6.53 (d, J = 8 Hz, 1H), 6.58 (s, 1H), 6.88 (d, J = 8 Hz, 1H), 8.07 (s, 1H); MS 138 (17), 135 (16), 120 (83), 92 (42), 91 (100), 65 (18), 51 (16), 40 (17), 39 (21).

2-Hydroxy-5-methylbenzenemethanol (**28**). Purification by flash chromatography using 20% (v/v) EtOAc–Hex gave **28** as a white solid: mp 102–103°C; lit 105°C (Dunning, et al., 1936); R_f 0.35 [45% (v/v) EtOAc–Hex]; IR (mineral oil) 3550–2500 s, 1000 s, 810 s; [^{1}H]NMR ($CDCl_3$–acetone-d_6) 2.22 (s, 3H), 2.76 (br s, 1H), 4.72 (s, 2H), 6.60–7.00 (m, 3H), 8.02 (br s, 1H); MS 138 (21), 136 (13), 120 (58), 92 (33), 91 (100), 77 (14), 65 (14), 51 (13), 39 (13).

2-Hydroxy-6-methylbenzenemethanol (**29**). Purification by flash chromatography on a 1 × 15-cm column using 20% (v/v) EtOAc–Hex gave **29** as a white solid: mp 104°C sharp; lit 80°C (Dunning et al., 1936); R_f 0.25 [35% (v/v) EtOAc–Hex]; IR (mineral oil) 3500–2800 m, 970 m, 775 m, 735 m;

[^{1}H]NMR (acetone-d_6) 2.22 (s, 3H), 4.40 (t, J = 5 Hz, 1H), 4.81 (d, J = 5 Hz, 2H), 6.58 (d, J = 8 Hz, 1H), 6.62 (s, 1H), 6.94 (d, J = 8 Hz, 1H), 8.61 (s, 1H); MS 138 (47), 121 (19), 120 (64), 92 (46), 91 (100), 51 (14), 40 (11).

1-(2-Acetyloxyphenyl)ethanone (**52**). Purification by flash chromatography on a 3 × 20-cm column using 20% (v/v) EtOAc–Hex gave **52** as a light yellow solid: mp 83–85°C; lit 80–81°C (Wittig and Schulze, 1931); R_f 0.45 [35% (v/v) EtOAc–Hex]; IR (mineral oil) 1755 s, 1676 s, 765 s; [^{1}H]NMR ($CDCl_3$) 2.33 (s, 3H), 2.53 (s, 3H), 6.94–7.83 (m, 4H); MS 178 (8), 136 (75), 121 (100), 43 (76).

1-(2-Hydroxyphenyl)ethanone (**53**). Purification by flash chromatography on a 1 × 30-cm column using 20% (v/v) EtOAc–Hex gave **53** as a light yellow oil: lit bp 218°C (Auwers et al., 1925); R_f 0.58 [35% (v/v) EtOAc–Hex]; IR (neat) 3400–2500 m, 1638 s, 748 s; [^{1}H]NMR ($CDCl_3$) 2.60 (s, 3H), 6.70–7.73 (m, 4H), 12.24 (s, 1H); MS 136 (54), 121 (100), 93 (24), 65 (21), 43 (16).

1-(2-Acetyloxy-3-methoxyphenyl)ethanone (**58**). The synthesis gave **58** as a light yellow oil: lit reported as a brown oil (Holshouser and Kolb, 1986); R_f 0.40 [35% (v/v) EtOAc–Hex]; IR (neat) 1755 s, 1675 s, 775 s; [^{1}H]NMR ($CDCl_3$) 2.34 (s, 3H), 2.52 (s, 3H), 2.81 (s, 3H), 6.91–7.39 (m, 3H); MS (no M^+), 166 (74), 151 (100), 43 (80).

1-(2-Hydroxy-3-methoxyphenyl)ethanone (**59**). Purification by flash chromatography gave **59** as lemon yellow needles: mp 52–53°C; lit 51.8–53°C (Amstutz, 1949); R_f 0.50 [35% (v/v) EtOAc–Hex]; IR (mineral oil) 3500–2500 s, 1640 s, 775 s, 730 s; [^{1}H]NMR ($CDCl_3$) 2.62 (s, 3H), 2.88 (s, 3H), 6.67–7.10 (m, 2H), 7.27 (d, J = 2, 8 Hz, 1H), 12.58 (s, 1H); MS 166 (61), 151 (100), 108 (14), 77 (11), 43 (25).

Preparation of Artificial Seeds for Field Bioassays

Compounds prepared for field bioassays were placed on 3 to 5-mm lengths of molecular sieves (1.5-mm-diameter 3 Å pellets). The sieves, or artificial seeds, were light weight, chemically inert with the test compounds, and approximately similar in size and shape to the seeds of ant-garden epiphytes.

Test compounds were deposited on the sieves by dissolving them in solvents (see tables below) with high volatility and then removing the solvents in vacuo. One unintentional source of error that plagued the coating process was the efficiency of applying the test compounds to the sieves. In 1984 and September 1988, a 25-ml round-bottom flask was used when coating about 1.5 g of sieves with each test compound. Under these conditions, an average coating efficiency of 93% (standard deviation 5.3%) was obtained when applying 20–30 mg of the various test compounds per gram of sieves, while an average coating efficiency of 48% (standard deviation 16.1%) was obtained when apply-

ing 4 mg of test compound per gram of sieves. In 1985 and February 1988, use of 50- and 100-ml round bottom flasks in the coating process probably reduced coating efficiency. Although this methodological difference may have introduced some inconsistency between trials in different field seasons, it is unlikely that this inconsistency affected the conclusions reached in our investigations.

Coated artificial seeds were placed in screw-cap vials, sealed with paraffin wax, and stored inside a closed plastic container until used in the field. In all years except year 1, we packaged seeds of each test compound in each of two vials. Because volatiles might be lost when vials are opened, we tried to complete trials with each compound within 48 hr after opening. For additional tests, we used material from the replicate vials. Finally, control seeds were made by placing sieves in appropriate solvents, removing the solvents in vacuo, and then sealing and storing the seeds in a manner identical to that for the test compounds.

In 1984 and 1985, two concentration ratios of test compounds to sieves were chosen to represent "high" and "low" concentrations on a weight/weight basis. "High" concentration was equivalent to 20 mg of test compound per gram of sieves. This choice was based on the approximate yield of crude oils extracted per gram of dry epiphyte seeds, plus a margin for error due to evaporation during transport, storage, and field tests. "Low" concentration was set at 0.2 mg test compound per gram of sieves, based on the approximate contribution a single extract component might make if 10 components of equal value were present, plus the margin of error for evaporation. In 1988, we varied concentrations in more complex ways (see below).

Finally, vials of test compounds were labeled only with numbers, whose corresponding identities were not known to the field experimentalist. In the field trials during the final year, uncoated seeds were included among the numbered test compounds.

Field Bioassays

Field tests were conducted at two research sites in the Department of Madre de Dios, Peru. These sites were: (September and October of 1984, 1985, and 1988) Cocha Cashu Biological Station on the Manu River in the Manu National Park (11°52′ S, 71°22′ W, elevation $\simeq$400 m) and (February and March of 1988) Tambopata Reserve at the junction of the Tambopata and Torre Rivers (12°50′ S, 69°70′ W, elev. $\simeq$290 m).

Ant responses to artificial seeds were measured by setting out experimental or test seeds (coated with test compounds and solvents) and control seeds (coated with solvent only) on opposite sites of small plastic weighing dishes (8 $\times$ 8 cm), taped to branches in areas of high *Ca. femoratus* activity approximately 0.5–3.0 m from focal ant gardens. "Walls" of masking tape partitioned the

trays into two halves and prevented experimental and control seeds from mixing. Seeds were added to the trays only after ants had discovered the trays and ceased to react excitedly to them (~5–10 min after introduction). Equal numbers (eight in early years, 10 in the final year) of control and experimental seeds were introduced to the trays simultaneously. Individual ant workers tended to travel over the entire tray, pushing and prodding seeds and contacting the two seed types approximately simultaneously.

Because worker behaviors were not consistent among years, responses were scored differently in the different years. In 1984, workers often carried certain seed types into their nests, and we scored the seeds as either "removed" (to the nest) or "remaining" after 30 to 45-min trials. Trials were discontinued after 30 min if all test seeds had been removed from the tray or if ants had ceased visiting the tray. Otherwise, the trials were continued for 45 min.

In later years, carrying was observed only rarely, and test compounds were scored as either "stimulants," "repellents," or "inert," depending on the responses they elicited from ants. Stimulants elicited rapid and excited running, release of mandibular gland products with a violent jerking movement (see Davidson, 1988), aborted attempts at carrying, and even attacks (usually only from major and media size classes of workers). Repellents led to avoidance of seeds after approaches to various degrees of proximity or contact. Strong repellents sometimes prevented ants from entering the seed trays. Inert compounds elicited no noticeable response after brief contact, sometimes including antennation and brief handling. Especially in 1984, when seed carrying was observed, we tested the seeds in multiple colonies, including colonies in different ant-garden aggregations where colonies were not directly related through colony fissioning (see Davidson, 1988). During the 1984–1985 wet season, erosion of the riverbank carried away most of the ant gardens with which we had worked at Cocha Cashu. In subsequent years, we rotated our experiments among the few remaining ant gardens that could easily be accessed at this study site. After large numbers of experiments, major and media workers often began to discard the artificial seeds as soon as these were made available. This behavior was taken as a sign that colonies had become habituated to the trials, and we "rested" these colonies for at least two weeks before returning to them.

Ant responses to control and experimental seeds were compared using Fisher exact tests. In 1984, tests compared tallies of seeds removed versus seeds remaining after standard time trials. During the following years, when seed carrying was extremely rare, tests compared two categories of worker responses toward control and experimental seeds: no response (compounds inert) and response (including both stimulation and repulsion). By inspecting the raw data, significant responses then could be categorized as either stimulation or repulsion. When conducting large numbers of trials, we required $P \leq 0.001$ (1985)

or $P \leq 0.01$ (September–October 1988) for statistical significance, to reduce the probability of type I error in hypothesis testing.

Finally, in 1988, ant responses to known epiphyte seed compounds were tested with individual compounds or combinations of compounds, varying both absolute and relative concentrations. Field time was extremely limited during the February–March trials, and ant responses were characterized by verbal descriptions, rather than systematic data collection. In September and October of 1988, ant responses were recorded and tested as in earlier years.

RESULTS

Table 2 gives the results of the 1984 field tests with high concentrations of test compounds; low concentrations produced no detectable ant responses. Of the 15 test compounds, **5, 6,** and **10** were most effective in stimulating seed collection by *Camponotus* ants. Of these three substances, only **5** and **6** are known to occur naturally on the seeds of some ant-garden epiphytes, but seed carrying was consistent over different colonies and ant-garden aggregations only for compound **10.** In response to **10,** workers first handled seeds without noticeable reaction, but excitement and alarm gradually increased. Eventually, workers began pounding the trays with their open mandibles, appearing to release mandibular alarm pheromone. As these behavioral responses increased, greater numbers of workers were recruited to the trays. Interestingly, workers of *Cr. l. parabiotica* also recruited to **10** during one test, breaking off pieces of the molecular sieves and carrying these back to the nest. For compounds attractive to *Ca. femoratus*, workers visited the side of the tray housing experimental seeds in preference to the side with control seeds. Occasionally, excited workers contacted one another's mandibles.

The remaining compounds elicited only mild responses from *Camponotus*, with the exception that the two methyl esters (3-hydroxy-4-methoxybenzoic acid, methyl ester **21** and 4-hydroxy-3-methoxybenzoic acid, methyl ester **22**) each produced significant ant responses (seed carrying) in one test. Compound **15** (2-hydroxy-3-methoxybenzaldehyde) was strongly repellent to the ants at high concentration.

In seed trials at Cocha Cashu in 1985, low concentrations of test compounds elicited no response from *Camponotus* ants, and Table 3 records responses for high concentrations only. Because of early difficulties finding sufficiently active colonies, we include data only for the last trial with each compound. (Two trials were run for most but not all compounds.) During all of the 1985 experiments, only one seed was ever carried into a nest garden; this seed was coated with compound **10,** the same compound that had elicited carrying

TABLE 2. RESPONSES OF *Ca. femoratus* TO TESTS WITH ARTIFICIAL SEEDS IN 1984[a]

Compound (#) [source]	Solvent	Colony-Trial	Test
Monoterpenes			
(+)-Limonene (**6**) [A]	N	1,1-1	NS
		1,1-2	0.0007
		1,1-3	0.0001
		1,2-1	0.0007
		2,1-1	NS
Benzenemethanols			
2-Hydroxy-3-methoxybenzenemethanol (**10**) [P_1]	M	1,1-1	0.0001
		1,1-2	0.0007[c]
		1,2-1	0.0001
		1,2-2	0.0007
		2,1-1	0.0007
3-Hydroxy-4-methoxybenzenemethanol (**11**) [A]	M	1,1-1	NS
		1,2-1	NS
4-Hydroxy-3-methoxybenzenemethanol[b] (**12**) [A]	M	1,1-1	NS
		1,1-2	NS
		2,1-1	NS
3-Ethoxy-4-hydroxybenzenemethanol (**13**) [I]	M	1,1-1	NS
		1,2-1	NS
		2,1-1	NS
Benzaldehydes			
2-Acetyloxy-3-methoxybenzaldehyde (**14**) [P_2]	M	1,2-1	NS
		1,2-2	NS
		2,1-1	NS
2-Hydroxy-3-methoxybenzaldehyde (**15**) [A]	D	1,1-1	NS
		1,2-1	NS
		1,2-1	NS
		2,1-1	NS
3-Ethoxy-4-hydroxybenzaldehyde (**16**) [M]	D	1,1-1	NS
		1,2-1	NS
		2,1-1	NS
3-Hydroxy-4-methoxybenzaldehyde (**17**) [C]	D	1,1-1	NS
		1,2-1	NS
		2,1-1	NS
4-Acetyloxy-3-methoxybenzaldehyde (**18**) [I]	M	1,1-1	NS
		2,1-1	NS
4-Hydroxy-3-methoxybenzaldehyde[b] (**5**) [A]	M	1,1-1	0.01282
		1,2-1	0.03846
		2,1-1	NS
Benzoic acids			
3-Hydroxy-4-methoxybenzoic acid (**19**) [A]	M	1,1-1	NS
		1,2-1	NS
4-Hydroxy-3-methoxybenzoic acid[b] (**20**) [A]	E	1,2-1	NS
		1,4-1	NS
		2,1-1	NS
		2,1-1	NS

TABLE 2. Continued

Compound (#) [source]	Solvent	Colony-Trial	Test
Benzoic acid esters	M	1,1-1	0.0130
3-Hydroxy-4-methoxybenzoic acid, methyl ester (**21**) [I]		1,2-1	NS
		1,4-1	NS
		2,1-1	NS
4-Hydroxy-3-methoxybenzoic acid, methyl ester (**22**) [P_2]	M	1,1-1	NS
		1,2-1	NS
		2,1-1	NS
		1,4-1	0.0035

[a] Significance levels reported where number of test seeds carried back to the nest > number of control seeds retrieved (1-tailed Fisher exact probability ≤ 0.05, $N = 8$ test and 8 control seeds available simultaneously). Colony designations give number of ant-garden aggregation, followed by colony identification and trial numbers. Sources of compounds: A = Aldrich Chemical Co.; C = City Chemical Co.; I = In-house chemical previously prepared by students; P_1 = prepared, see Seidel et al. (1990); P_2 = prepared, see Methods and Materials. Solvents used to coat the seeds (sieves) as discussed in Methods and Materials: D = dichloromethane; M = methanol; E = ether.

[b] Compound occurs on the seeds of *Vanilla planifolia* (Klimes and Lamparsky, 1976).

[c] *Cr. l. parabiotica* also recruiting to tray.

during the previous year. Two observations suggested the need to use alternative solvents and investigate lower concentrations of test compounds. First, dichloromethane, the solvent used most frequently in these trials, may have contained impurities; workers were sometimes mildly repelled by control as well as test seeds, and they often appeared unable to hold onto seeds for more than a brief time. Second, concentrations of many active compounds were apparently too high, as evidenced by very strong ant responses and their greater difficulty holding test seeds, in comparison to control seeds.

Although Table 3 distinguishes repellent compounds from stimulants, 10 compounds classed as stimulants elicited different responses from the different worker castes. Minor workers tended to alarm and run excitedly through the seed tray, whereas reactions of majors and medias were more ambiguous. These larger ants frequently appeared to stalk defensively and aggressively with mandibles open. Members of the two larger castes also discarded many seeds (these were usually control seeds), perhaps because of the greater difficulty the ants had in handling seeds coated with test compounds. Discards were not included in the data set, because they could not be scored unambiguously as stimulants or repellents, and they tended to occur at the ends of tests, before we moved the experiments to new colonies.

The data obtained from these trials do reveal a response pattern related to the known ant-garden epiphyte seed components. Specifically, compounds **1, 6** and 1-(2-hydroxy-6-methylphenyl)ethanone, **3** (all found on ant-garden epiphyte seeds), are among the 20 most active stimulant compounds (with $P \leq$

TABLE 3. RESPONSES OF *Ca. femoratus* TO TESTS WITH ARTIFICIAL SEEDS IN 1985[a]

Test compound (#) [source]	Colony-Trial	*P*
Monoterpenes		
(+)-Limonene (**6**) [A]	UU	**s
(−)-α-Pinene (**7**) [A]	61	NS
(+)-β-Pinene (**8**) [A]	51	NS
Citral A and B (**23**) [J]	33	*r
(−)-Citronellal (**24**) [A]	3U	NS
Benzenemethanols		
2-Hydroxybenzenemethanol (**25**) [P_2]	61	NS
2-Hydroxy-3-methylbenzenemethanol (**26**) [P_2]	51	**r
2-Hydroxy-4-methylbenzenemethanol (**27**) [P_2]	3U	*sd
2-Hydroxy-5-methylbenzenemethanol (**28**) [P_2]	61	NS
2-Hydroxy-6-methylbenzenemethanol (**29**) [P_2]	51	NS
2-Hydroxy-3-methoxybenzenemethanol (**10**) [P_1]	71	**sd
3-Hydroxy-4-methoxybenzenemethanol (**11**) [A]	32	NS
4-Hydroxy-3-methoxybenzenemethanol (**12**) [A]	3U	*s
Benzaldehydes		
Benzaldehyde (**30**) [A]	UU	NSs
2-Hydroxy-3-methoxybenzaldehyde (**15**) [A]	36	*sd
2,3-Dimethoxybenzaldehyde (**31**) [A]	61	NS
3-Hydroxy-4-methoxybenzaldehyde (**17**) [C]	UU	**sd
4-Hydroxy-3-methoxybenzaldehyde (**5**) [S]	51	NS
Benzoic acids		
Benzoic acid (**32**) [B]	3U	**s
Cinnamic acid (**33**) [A]	11	*s
Hydrocinnamic acid (**34**) [M]	11	NS
2-Aminobenzoic acid (**35**) [E]	61	NS
2-Hydroxybenzoic acid (**36**) [M]	UU	NS
3-Hydroxybenzoic acid (**37**) [A]	34	NS
4-Hydroxybenzoic acid (**38**) [A]	61	NS
2-Hydroxy-3-methylbenzoic acid (**39**) [A]	61	NS
2-Hydroxy-4-methylbenzoic acid (**40**) [A]	41	NS
2-Hydroxy-5-methylbenzoic acid (**41**) [A]	61	NS
2-Hydroxy-6-methylbenzoic acid (**42**) [P_1]	3U	NS
2-Hydroxy-3-methoxybenzoic acid (**43**) [I]	61	NS
2-Hydroxy-4-methoxybenzoic acid (**44**) [A]	43	NS
2-Hydroxy-5-methoxybenzoic acid (**45**) [A]	32	NS
3-Hydroxy-4-methoxybenzoic acid (**19**) [A]	41	NS
4-Hydroxy-3-methoxybenzoic acid (**20**) [A]	51	**sd
2,4-Dimethoxybenzoic acid (**46**) [A]	31	NS
Benzoid acid esters		
2-Hydroxybenzoic acid, methyl ester (**47**) [A]	61	**r
2-Hydroxy-3-methylbenzoic acid, methyl ester (**48**) [P_1]	61	**sd
2-Hydroxy-4-methylbenzoic acid, methyl ester (**49**) [P_1]	61	**r
2-Hydroxy-5-methylbenzoic acid, methyl ester (**50**) [P_1]	33	**sd
2-Hydroxy-6-methylbenzoic acid, methyl ester (**1**) [P_1]	3U	**sd
2-Hydroxy-3-methoxybenzoic acid, methyl ester (**51**) [I]	3U	NS

TABLE 3. Continued

Test compound (#) [source]	Colony-Trial	*P*
Phenylethanones		
1-(2-Acetyloxyphenyl)ethanone (**52**) [P_2]	7U	**sd
1-(2-Hydroxyphenyl)ethanone (**53**) [P_2]	3U	NS
1-(4-Hydroxyphenyl)ethanone (**54**) [A]	45	NS
1-(2-Hydroxy-3-methylphenyl)ethanone (**55**) [P_1]	UU	**sd
1-(2-Hydroxy-4-methylphenyl)ethanone (**56**) [P_1]	61	**s
1-(2-Hydroxy-5-methylphenyl)ethanone (**57**) [P_1]	61	**s
1-(2-Hydroxy-6-methylphenyl)ethanone (**3**) [P_1]	34	**sd
1-(2-Acetyloxy-3-methoxyphenyl)ethanone[b] (**58**) [P_2]	51	**r
1-(2-Hydroxy-3-methoxyphenyl)ethanone (**59**) [P_2]	51	*r
1-(2-Hydroxy-4-methoxyphenyl)ethanone (**60**) [A]	UU	**sg
1-(4-Hydroxy-3-methoxyphenyl)ethanone (**61**) [A]	33	NS
1-(2,4-Dihydroxyphenyl)ethanone (**4**) [A]	61	NS
1-(3,4-Dihydroxyphenyl)ethanone (**62**) [A]	61	NS
Phenols		
1,2-Dihydroxybenzene (**63**) [E]	UU	NS
1,3-Dihydroxy-5-methylbenzene (**64**) [E]	31	NS
1,3-Dihydroxy-5-methoxybenzene (**65**) [A]	31	NS
Eugenol (**66**) [MK]	3U	**s
Isoeugenol (**67**) [A]	3U	NS
Nitrogen-containing aromatics		
Piperic acid (**68**) [I]	41	NS
Piperine (**69**) [I]	UU	NS
Skatole[b] (**70**) [A]	61	NS

[a] Colony-trial designations (U = unknown, not recorded): first character = number of ant-garden aggregation or cluster; second character = identity of colony within cluster. Two-tailed Fisher exact probabilities significant at $P \leq 0.001$ (*) or ≤ 0.00005 (**) in 30- to 45-min trials with eight control and eight test seeds. Behavioral responses: s = stimulation (excitement, running, alarm, attack); d = dichotomous responses of workers (excitement, running, alarm) and soldiers (attack, or stalking and searching with mandibles open); r = repulsion from a distance or up close; g = massive alarm/recruitment and stalking by soldiers, most workers and soldiers eventually leaving bait trays and moving to the ground. This last behavioral response occurred only in response to 1-(2-hydroxy-4-methoxyphenyl)ethanone **60**, and occurred in a second trial with this compound. Sources of compounds and solvents as in Table 2; additional sources, B = Baker & Adamson; E = Eastman; J = J. T. Baker; S = Spectrum Chemicals; all seed samples (sieves) were coated as discussed in Methods and Materials, using dichloromethane as a solvent, except for **19** (methanol) and **68** (dichloromethane and acetone).

[b] Not checked for purity as discussed in Methods and Materials, used as received or prepared.

0.00005 in field tests). The relationship between ant activity and actual seed compounds is upheld by the pattern of the most active classes of compounds listed in Table 3. These are: (1) the benzoic acid esters (five of six tested), including 2-hydroxybenzoic acid, methyl ester **47** (found on some ant-garden epiphyte seeds, see Seidel et al., 1990), and **1** (the most widespread ant-garden

epiphyte compound identified) and its isomeric 2-hydroxy-methylbenzoic acid, methyl esters **48–51**; (2) the phenylethanones (eight of 13 tested), including **3** (an ant-garden epiphyte seed component) and its isomeric 1-(2-hydroxymethylphenyl)ethanones (**55–57**) and some structurally similar phenylethanones (see Table 3); (3) the benzenemethanols (four of eight tested), including **10** and the benzenemethanol produced by the reduction of **5.** Why the benzenemethanols are active remains a question, but the underlying pattern for all the active compounds tested in 1985 suggests that only compounds present on the ant-garden epiphyte seeds or closely related structural analogs are effective in eliciting ant responses.

Finally, one test compound [1-(2-hydroxy-4-methoxyphenyl)ethanone, **60**] elicited a unique behavioral response on the part of *Ca. femoratus*. Workers of all castes quickly left the bait tray and the area surrounding it and moved to the ground below the tray. This mass exodus to the ground also occurred in an earlier trial with a different colony.

Table 4 gives anecdotal observations (at the Tambopata Reserve in February–March 1988) of ant responses to actual seed compounds and combinations of compounds, as well as to repeat trials with compound **10.** Of a total of 130 seeds coated with test compounds and offered to the ants of a single colony (13 experiments × 10 seeds per experiment), only one seed was taken into the nest. This seed carried compound **1** alone at a concentration of 30 mg/g molecular sieves. The carrying event occurred without any strong alarm response or excitement on the part of the worker. The worker picked up a seed immediately after entering the tray and then carried it approximately 2 m to its nest. Unfortunately, this trial was interrupted by rain. Seeds stored in the original vial overnight elicited excitement and alarm but no carrying on the following day. Handling of tests seeds was greatest for those coated with **1** alone or **10** alone, and ants were repelled consistently when **2** was mixed with **1,** and especially at high concentrations of **2.**

Finally, during the September–October trials at Cocha Cashu in 1988, few seeds were carried by the ants (Table 5). In response to a combination of **1** and **5** (20 mg each per gram of molecular sieves), one worker carried a control seed a short distance toward the nest, and a second grasped a nestmate by an antenna and carried the worker back to the nest. Similarly, a control seed was carried a distance of 2 m toward the nest during a trial with test compounds imitating the combination of seed compounds isolated from *Peperomia macrostachya* in 1984. In a test with blank seeds (lacking both solvent and test compounds), one such seed was carried back to a nest garden, but several repetitions of tests with seeds from this vial elicited no further carrying.

Most test compounds and combinations of compounds were significant stimulants or repellents (Table 5). The most widely distributed epiphyte seed compound, **1,** stimulated extreme excitement and considerable handling at the

TABLE 4. RESULTS OF FIELD TESTS IN FEBRUARY–MARCH 1988[a]

Relative ratios of test compound(s)	Concentration (mg/g sieves)	Description of ant response
1 only	10	No response.
1 only	30	Excitement, release of mandibular alarm pheromone, one seed carried immediately into the nest. Workers handled but released other seeds. Soldiers grabbed and released seeds aggressively.
1:**2** at 1:9	20	Repellent. Ants deserted tray.
1:**2** at 3:1	20	Running, excitement, mild repellent.
1:**2** at 3:1	30	Repellent stimulating ant aggression.
1:**2** at 1:9	10	Rain interrupted trial.
1:**2** at 1:19	30	Rain interrupted trial.
1:**2** at 9:1	30	Great excitement, running, release of alarm pheromone, no recruitment, ants eventually left tray (mildly repellent?).
1:**2** at 9:1	10	Excitement, running, seeds grasped and released.
1:**2** at 9:1	20	Aggression, release of alarm pheromone, pounding of mandibles on tray, no recruitment. Mildly repellent after contact.
10 only	10	Handling, no excitement.
10 only	30	Handling, no excitement.

[a] All seed samples (sieves) were coated as discussed in Methods and Materials, using methanol as solvent.

lowest concentration, but workers were able to hold the seeds only briefly. At higher concentrations this compound also led to much handling but was mildly repellent after contact. In contrast, the next most widely distributed epiphyte seed compound, **2**, was distinctly repellent at all concentrations tested and probably contributed to the repellency of all combinations of compounds in which it was included. Combinations of compounds including high concentrations of **1** but lacking **2** were also mostly repellent. Of two less ubiquitous epiphyte seed compounds, **3** was mildly repellent at low concentrations and strongly repellent at high concentrations, while 1-(2,4-dihydroxyphenyl)ethanone, **4**, elicited no detectable response at any concentration but was handled a great deal at 4 mg/g sieves.

Finally, compound **10**, which had elicited much carrying in previous trials, produced no significant results in tests reported in Table 5. The lack of signif-

TABLE 5. RESULTS OF FIELD TESTS IN SEPTEMBER–OCTOBER 1988[a]

Test compound(s)	Concentration (mg/g sieves)	Description of ant response
Pure compounds		
1	4.0	Much contact and handling but difficulty grasping seeds, pounding of mandibles on tray, extreme excitement, handling of test seeds exceeded that of control seeds.
1	20.0[b]	Workers excited, soldiers searching for sources of compound, repellent at close range.
1	40.0[b]	Much handling, mild repellent.
2	4.0[b]	Repellent, excitement, running, aggression.
2	20.0[b]	Strong repellent.
2	40.0	Excitement, running, aggression, avoidance.
3	4.0[b]	Mild repellent.
3	20.0	Ants attracted from distance, repelled close up.
3	40.0[b]	Repellent before (usually) and after contact.
4	4.0	No detectable response, much handling of control and test seeds.
4	20.0	No detectable response.
4	40.0	No detectable response.
5	4.0	All test seeds rapidly discarded.
5	20.0[c]	Excitement, running, alarm, handling of test seeds exceeded that of control seeds.
5	40.0[b]	Excitement, running, alarm.
10	4.0[c]	Much handling of test seeds, exceeds that of control seeds.
10	20.0	No noticeable response.
10	40.0[b]	Ants frantic near test seeds, control seeds discarded, handling of test seeds exceeded that of control seeds.
Solvent only seeds		No detectable response, handling of test seeds exceeded that of control seeds.
Simple mixtures of compounds		
1:2	20.0:20.0[b]	Repellent after recruitment.
2:5	20.0:4.0[b]	Strong repellent.
1:2	4.0:4.0[b]	Repellent.
1:5	4.0:20.0[b]	Mildly repellent after attraction from a distance.
2:5	4.0:4.0[b]	Mild repellent.

TABLE 5. Continued

Test compound(s)	Concentration (mg/g sieves)	Description of ant response
Simple mixtures of compounds		
1:5	20.0:20.0[b]	Bursts of extreme excitement, one worker grasped another by antennae and carried it to the nest.
1:5	4.0:4.0[b]	Mild repellent.
1:2	4.0:20.0[b]	Strong repellent.
1:2	20.0:4.0[b]	Excitement, some avoidance, seeds grasped then sharply released.
1:5	20.0:4.0[b]	Aggressive but failed attempts to grasp test seeds.
2:5	20.0:20.0[b]	Repellent before (usually) or after contact.
2:5	4.0:20.0[b]	Excitement, running, aggression.
Mixtures imitating natural combinations on epiphyte seeds		
Peperomia macrostachya (1989 seed analysis)		
1	20.0	Extreme excitement, running, attempts to grasp but seeds quickly released, pounding of mandibles on tray, control and test seeds eventually discarded.
2	3.7	
4	0.8	
5	8.2	
Peperomia macrostachya (1985 seed analysis)		
1	20.0[b]	Mild repellent.
4	4.5	
5	4.5	
Neoregelia sp.		
1	20.0[b]	Strong repellent.
3	15.3	
4	15.1	
5	1.8	
Streptocalyx longifolius		
1	20.0[b]	Repellent.
2	0.3	
3	0.3	
4	1.9	
5	0.3	
Epiphyllum phyllanthus (1984 seed analysis)		
1	20.0[b]	Repellent, workers alarm, soldiers drag test seeds short distances and discard control seeds.
2	9.1	
3	4.1	
Condonanthe aleana		
1	20.0[b]	Mild repellent.
5	2.4	

TABLE 5. Continued

Test compound(s)	Concentration (mg/g sieves)	Description of ant response
Markei ulei		
1	20.0[b]	Excitement, running, mild repellent.
3	1.1	
4	0.3	
5	0.3	
Anthurium gracile		
1	12.0[b]	Repellent, control seeds discarded.
2	20.0	
4	6.9	

[a] All seed samples (sieves) were coated as discussed in Methods and Materials, using ether as the solvent.
[b] Test seeds stimulate or repel ants more than control seeds.
[c] Test seeds handled significantly more than control seeds within responses scored as no response (1-tailed Fisher exact tests with $P \leq 0.01$).

icance is a consequence of the manner in which data were scored and does not fairly represent the compound's activity. Thus, although seed handling was treated in the statistical comparisons as no response, it is also instructive to look at whether ants handled test seeds or control seeds at greater frequency. In the 40 trials, seeds with test compounds were handled more than control seeds in only five experiments, including two experiments with **10** (40 mg/g sieves and, especially 4 mg/g sieves) and one experiment each with actual epiphyte seed compounds **5** (20 mg/g sieves), **1** (4 mg/g sieves) and **4** (4 mg/g sieves). In the final such case, handling of test seeds exceeded that of control seeds by only one seed when the test seeds were coated with solvent only. Seed handling was represented significantly more in the no-response category for test seeds than for control seeds only for compounds **5** (20 mg/g sieves) and **10** (mg/g sieves) (Table 5). Unfortunately, data taken during earlier trials were not taken in such a way as to allow similar comparisons.

DISCUSSION

Four independent sets of field experiments in southeastern Peru together suggest that: (1) particular volatile compounds can and do elicit seed carrying by ants, (2) chemical structures similar to those of actual epiphyte seed compounds are particularly active in inducing seed carrying and in stimulating worker excitement and alarm, and (3) ant responses can depend on the concentrations of test compounds and the identities of worker castes.

Although few artificial seeds were collected by ants after the initial set of trials in 1984, carrying of test seeds occurred principally for seeds coated with actual epiphyte seed compounds or their structural relatives (Table 1) (Seidel et al., 1990). Compounds in the first category include: **1** (1985 and 1988), **5** (1984), **6** (1984), and a combination of both **1** and **5** (September–October 1988). Of 70 compounds tested, **10** was the most active and consistent in eliciting seed carrying by ants. Although **10** has not been isolated from the seeds of any ant-garden epiphyte (Seidel et al, 1990), it does have a 1,2,3-substitution pattern like compound **1,** and adjacent hydroxyl and methoxy groups like compound **5**. Together, these structures may stimulate the sensory apparatus of ants and account for the otherwise anomalous response of workers to **10.** Other compounds such as **21** and **22** (stimulating seed carrying in 1984) bear a more direct relationship to ant-garden epiphyte components **1** and **5.** Compound **21** is the methyl ester equivalent (i.e., analog to **1**) of compound **5,** while compound **22** is the isomer of compound **21,** with the hydroxyl and methoxy groups switched.

In contrast, ant-garden epiphyte compounds **2** and **3** repelled *Ca. femoratus* at all concentrations. Although compound **2** is structurally unrelated to the other epiphyte seed compounds, compound **3** has features in common with these substances. The presence of **3** and related phenylethanones as defensive phermones of several *Hypoclinea* species (Blum et al., 1982) may explain its repellency to *Ca. femoratus*. Overall, there is compelling evidence that compounds **2** and **3** are not attractants that stimulate the collection of epiphyte seeds.

Undoubtedly, test seeds emitted much purer signals than ants experience in their contact with actual epiphyte seeds, even when combinations of test compounds were employed. Together with the lack of ancillary (e.g., tactile) stimuli, the isolation of one or a few chemical stimuli from normal background olfactory stimuli may help to explain why ants carried seeds with compounds similar, but not identical, to actual epiphyte seed compounds. Occasional anomalous ant behaviors support the hypothesis that test seeds may have emitted sensory stimuli that were, in effect, too pure. Thus, when presented with seeds coated with actual epiphyte seed compounds, ants infrequently transported not only blank seeds (1988) and control seeds (1984 and (1988) from the same tray, but even a fellow worker (1988).

Ant responses to test compounds often varied with concentration (Table 5), but behaviors also varied across years in response to purportedly identical concentrations of test compounds (Tables 2–5). Across years, for example, worker responses to **10** ranged from strong stimulation but little handling (1985), to significant handling in relation to control seeds (1988), to actual seed transport to nest gardens (one seed in 1985 and all eight seeds for each of five trials with three colonies in 1984). Within years (Table 2 and other data not presented here), worker responses were similar across different colonies with this as well as other compounds.

The most parsimonious explanation for this pattern of results is unintended variation in the concentrations of test compounds actually present on the artificial seeds. After field trials in February–March 1988, we noted that considerable quantities of seed compounds remained on the inside walls of flasks in which seeds had been coated with solvent and test compounds. In preparing seeds for trials in September–October 1988, we coated seeds in a smaller flask (with less surface area), as we had in 1984, and took extreme care to achieve consistent, reproducible concentrations. It is likely then that actual compound concentrations for seeds reported as 4 mg/g sieves in later trials (Table 5) exceeded those reported as 10 mg/g sieves in earlier trials (Table 4). This may explain, for example, why 4 mg/g sieves of compound **10** appeared to be too concentrated for ants to handle, but 10 mg/g sieves in earlier tests elicited no response, including no significant handling.

Because our "high" concentration levels were tied to crude extract concentrations (rather than to specific compounds within the mixtures), these concentrations may regularly have been too high, perhaps explaining the generally high levels of excitation in the ants across our experimental trials. However, in November 1989, a brief screening of actual seed compounds (alone and in combination) at concentrations precisely determined to be intermediate between the high and low concentrations reported in Table 5 elicited no consistent carrying behavior. Despite the problems in obtaining precise concentrations of test compounds on sieves, it seems likely that we tested concentrations within the broad ranges in which compounds are found naturally on epiphyte seeds (Seidel et al. 1990). Thus, the fact that ants carried seeds so seldom is more likely to be explained by the absence of appropriate combinations of chemical and olfactory cues than by completely inappropriate concentrations of test compounds.

Although we still lack conclusive evidence that the compounds stimulate seed carrying, ant responses to compound **1** (and structurally similar compound **10**) appear significant. In fact, circumstantial evidence suggests that **1** could have stimulated seed carrying during the early evolutionary history of ant gardens. At that time, seeds may have been olfactory mimics of male brood, since **1** is known to occur in mandibular glands of males in several *Camponotus* species and appears in trace quantities in *Ca. femoratus* (reviewed in Seidel et al., in 1990). Even if this were so, it would not rule out other potential roles for seed compounds. Many of the volatile epiphyte seed compounds have antiseptic properties that might mediate interactions of epiphytes or ants with microbial pathogens in their environments (Seidel et al., 1990). Moreover, phenolic acids similar to the seed compounds most attractive to ants are known to affect seed germination rates in some plant species (Kuiters, 1989). The widespread distribution of volatile seed compounds among ant-garden epiphytes could be related to any or all of these factors.

Finally, although it may be tempting to conclude that taxonomically unrelated ant-garden epiphytes have evolved their seed chemistry convergently due

to symbiotic interactions with the same ants, we again call attention to two alternative hypotheses (Seidel et al., 1990). First, plants could have evolved identical compounds in response to unrelated selection pressures, but become concentrated in a single environment, because ants responded selectively to particular seed chemicals. Second, seeds of ant-garden epiphytes might possibly have acquired their compounds by infection with some symbiotic microflora of these social and colonial insects. Additional research will be necessary to distinguish between these alternatives.

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Editors' Note

This special issue is devoted to the main contributions at the International Symposium: Semiochemicals and Pest Control—Prospects for New Applications, which was held in Wageningen, The Netherlands, October 16–20, 1989. The editors are grateful to Drs. L.M. Schoonhoven, A.K. Minks, J.C. van Lenteren, and M. Dicke, who hosted the conference and served as guest editors. We thank the editors and all others who participated in the review process.

We acknowledge the cooperation of Plenum Publishing Corporation in producing extra copies of this issue to accommodate symposium registrants and others interested in this symposium.

R.M. Simeone
J.B. Silverstein
Editors
Journal of Chemical Ecology

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Journal of Chemical Ecology, Vol. 16, No. 11, 1990

Foreword

Proceedings of the International Symposium:
Semiochemicals and Pest Control—Prospects for New Applications

(October 16–20, 1989, Wageningen, The Netherlands)



Since the first identification of an insect sex pheromone (1959), chemical and biological studies on these semiochemicals have increased dramatically. Lepidopteran sex pheromones have received and still receive the most attention in the context of semiochemicals and pest control. This has led to commercial application of lepidopteran sex pheromones. Allelochemicals have become another focal point in semiochemical research, but there are no commercial applications to date.

During the symposium, the state of the art was evaluated and possibilities for the application of semiochemicals other than lepidopteran sex pheromones were discussed. Contributions concentrated on fundamental research, but also referred to applications. Among the organisms discussed were herbivores, vectors, pollinators, and natural enemies of pestiferous arthropods. The main subjects were (1) pheromones (excluding lepidopteran sex pheromones), (2) allelochemicals affecting pestiferous arthropods, (3) allelochemicals affecting beneficial arthropods, and (4) semiochemicals and learning. Each section consisted of two or three key introductions, a restricted number of additional papers, and a poster session; ample time was reserved for discussion.

Each invited speaker was asked to address the following questions:

1. What is known about semiochemicals in the respective fields?
2. Which aspects of current knowledge have led to application in pest control?
3. Which applications are to be expected?
   a. in the short term (within 5–10 years)?
   b. in the long term (after 10 years)?
4. What determines whether application will (eventually) be successfully carried out?

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The last two questions are especially important, although we realize that these are also the most difficult ones to answer. However, even when no applications are envisaged, this information is significant.

In this issue of the *Journal of Chemical Ecology*, the main contributions are presented, including ideas about application of semiochemicals in pest control. This relates to both toxins and information-conveying chemicals.

M. Dicke
J.C. van Lenteren
A.K. Minks
L.M. Schoonhoven
Guest Editors

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APHID SEMIOCHEMICALS—A REVIEW, AND RECENT ADVANCES ON THE SEX PHEROMONE

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Abstract—The alarm pheromones are known for many species of aphids, and methods of using the synthetic pheromone to improve control of aphids by contact insecticides and biological agents have been devised. Highly active analogs have been prepared and plant-derived synergists identified. Laboratory studies on compounds obtained by chemical modification of the alarm pheromone and antifeedants derived from non-host plants have led to successful field trials against aphid-borne virus diseases in crops. Strategies for biotechnological production of aphid semiochemicals are described. The sex pheromones for a number of aphids have recently been identified. Further studies on the production, perception, and interspecific attraction of the pheromone components are described and possible uses for the sex pheromone are discussed.

Key Words—Aphid, *Megoura viciae*, *Schizaphis graminum*, *Acyrthosiphon pisum*, *Aphis fabae*, *Myzus persicae*, Hemiptera, Aphididae, pheromone, alarm, sex, antifeedant, attractant, host plant, electrophysiology, single-cell recording, GC-MS.

INTRODUCTION

Review of Aphid Semiochemicals

Aphids are the main insect pests of northern European agriculture and are also important pests in horticulture, causing damage to crops either directly by feeding or by transmitting plant virus diseases. In view of the increasing problem

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of insecticide resistance (Ffrench-Constant et al.,1988) and in order to reduce hazards arising from use of insecticides, it is important to consider aphid semiochemicals as a means of providing new control methods.

Chemicals that influence the behavior of aphids have been studied for many years, but a major breakthrough was achieved in the early 1970s, with the simultaneous chemical identification by several groups of the alarm pheromone for a number of species (Bowers et al., 1972; Edwards et al., 1973; Wientjens et al., 1973). This pheromone was shown to comprise the sesquiterpene hydrocarbon, (*E*)-β-farnesene. Although it was clearly demonstrated that this material alone would not be useful in crop protection (Calabrese and Sorensen, 1978; Hille Ris Lambers and Schepers, 1978), it was subsequently shown that the increased mobility caused by synthetic (*E*)-β-farnesene improved pick-up of contact pesticides and fungal pathogens (Griffiths and Pickett, 1980, 1987), and collaborative work in China has shown that addition of (*E*)-β-farnesene to a commercial formulation of fenvalerate (Sumicidin) improved kill of the peach-potato aphid, *Myzus persicae*, in the field (Zhang, Z.-N., unpublished results). However, field trials in the U.K. gave erratic results, and as aqueous formulations were ineffective, application was restricted to the use of an electrostatic sprayer not yet commercially available. In order to improve the efficacy of the aqueous formulations, compounds that might enhance the activity of (*E*)-β-farnesene were sought.

Electrophysiological recordings from the primary rhinaria on the aphid antenna, coupled with high-resolution gas chromatography (GC), enabled the identification of synergists for the alarm pheromone of the turnip aphid, *Lipaphis erysimi* (Dawson et al., 1987a). These were found to be metabolites of glucosinolate precursors in the cruciferous host plants and the most active, allyl isothiocyanate, significantly improved the activity of an aqueous formulation of (*E*)-β-farnesene. This may allow use of conventional hydraulic sprayers against this pest in the field. To study the distribution of (*E*)-β-farnesene within the crop canopy, fluorinated analogs were prepared (Briggs et al., 1986). These had lower GC detection limits than the parent compound while still retaining biological activity.

(*E*)-β-farnesene is a common plant component, but is generally found together with other sesquiterpene hydrocarbons, e.g. (−)-β-caryophyllene, that act as potent inhibitors for the pheromonal activity. This allows the aphid to distinguish between (*E*)-β-farnesene produced by a plant and that produced alone, by aphids under attack (Dawson et al., 1984; Pickett et al., 1984). However, *Solanum berthaultii*, a close relative of the commercial potato (*S. tuberosum*), releases (*E*)-β-farnesene from specially adapted foliar hairs, thereby avoiding the inhibitory effect of other compounds produced by the leaf and eliciting an alarm effect in aphids attempting colonization (Gibson and Pickett, 1983).

S. berthaultii presumably overcomes the problems of high volatility and instability by producing the (*E*)-β-farnesene from biochemical precursors, e.g., farnesyl pyrophosphate, just prior to release. In investigating possible chemical precursors of the pheromone, derivatives were synthesized that showed more persistent activity against aphid feeding and colonization in the laboratory and against transmission of barley yellow dwarf virus in the field (Dawson et al., 1982, 1988a). This was the first demonstration of the use of a nontoxic antifeedant against an arable crop pest.

Aphid feeding is influenced by many plant components such as the crucifer glucosinolate sinigrin (Nault and Styer, 1972) and the hydroxamic acids of cereals (Argandoña et al., 1980), but here discussion is restricted to plant-derived antifeedants comprising lower terpenoids, which currently show promise for use against aphid pests. Some of the most active are the drimane sesquiterpenes from the African tree *Warburgia ugandensis* (Kubo et al., 1976) and several elegant synthetic routes to these compounds have been devised (Hollinshead et al., 1983; Jalali-Naini et al., 1983). Although a number of analogs were prepared, the natural compounds (−)-polygodial and (−)-warburganal showed greatest activity against aphid colonization or virus transmission (Gibson et al., 1982; Asakawa et al., 1988).

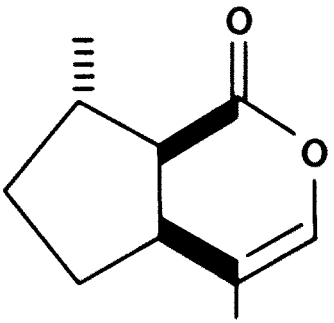
(−)-Polygodial also is produced by the temperate herbaceous weed waterpepper (*Polygonum hydropiper*). The antifeedant, extracted from the plant using liquified CO_2, was sprayed onto barley and gave a significant improvement in yield, demonstrating the principle of using a plant-derived antifeedant against aphid-mediated damage (Pickett et al., 1987).

Sesquiterpenoids are biosynthesized from farnesyl pyrophosphate, usually by cyclization and subsequent oxidation. The key step in the biosynthesis of the drimane antifeedants is the cyclization, which is likely to be controlled by one enzyme. Attempts are being made to isolate and clone the gene for this enzyme for expression in *E. coli*, to enable this organism to produce drimenyl pyrophosphate from endogenous farnesyl pyrophosphate (Dawson et al., 1989a). The drimenol would be a useful synthetic starting material for producing the drimane antifeedants. Furthermore, it may be possible to transfer this gene for expression in the vegetative parts of crop plants. Existing or genetically added oxidative pathways then could allow the plants to produce these benign crop protection agents.

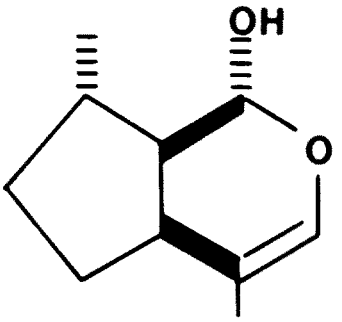
Some of the approaches to using the aphid semiochemicals described here could, within 5–10 years, achieve wide commercial use in crop protection by incorporation into integrated control methods. Genetic modification of crop plants to produce semiochemicals for control of aphids then may become more important. The recent advances in the identification and isolation of aphid sex pheromones also provide further possibilities for novel methods of aphid control.

Aphid Sex Pheromone: Recent Advances

The sex pheromones for a number of aphids have now been identified (Dawson et al., 1987b, 1988b, 1989b). In the early 1970s, Pettersson (1970, 1971) demonstrated that aphids in the *Schizaphis* genus employed a sex pheromone that was released from the hind tibiae of females to attract males. Later studies by Marsh (1972, 1975) also showed a sex pheromone for the vetch aphid, *Megoura viciae*. GC-coupled single-cell recordings from the secondary rhinaria on the third antennal segment of male *M. viciae*, and subsequent GC-MS, NMR, x-ray crystallography, and synthetic studies demonstrated that the sex pheromone comprised the (+)-(4a*S*,7*S*,7a*R*)-nepetalactone (I) and the (−)-(1*R*,4a*S*,7*S*,7a*R*)-nepetalactol (II) (Dawson et al., 1989b). These two compounds were shown to be present also in the sex pheromones of the black bean aphid *Aphis fabae*, the pea aphid *Acyrthosiphon pisum*, the peach-potato aphid *Myzus persicae*, and the greenbug *Schizaphis graminum* (Dawson et al., 1988b). Further chemical, behavioral, and electrophysiological studies are described and possible uses of the sex pheromone are discussed.



STRUCTURE I



STRUCTURE II

METHODS AND MATERIALS

Insects. Sexual aphids were produced at 16°C under short day conditions: *M. viciae*, *A. pisum*, and *A. fabae*, 12 hr light–dark; *S. graminum*, 9:15 hr light–dark.

Bioassay. Behavioral assays were done in a 100-m Perspex olfactometer similar to that described by Pettersson (1970), with a weak airstream directed towards the center from each of four side arms. Virgin oviparae were placed in a glass cylinder (5 × 25 mm) attached to the end of one of the side arms. Five males were introduced into the chamber and their positions noted every minute

for 15 min. Males in the center of the arena were not scored. The apparatus, maintained at 17°C, was lit from above by fluorescent lights and rotated through 120° every 5 min during the test. Assays were performed when the females were calling (i.e., releasing pheromone) and when the males were at their most active.

Electrophysiology. Single-cell recordings (SCR) from individual olfactory cells were made using tungsten microelectrodes. The indifferent electrode was placed in the first or second antennal segment and the recording electrode was implanted in the secondary rhinaria until impulses were obtained. SCR signals were amplified and recorded by standard methods and stimuli were applied as described previously (Wadhams et al., 1982).

Biological samples. Pentane extracts of female hind tibiae and air entrainment extracts form calling females were obtained as described previously (Dawson et al., 1987b, 1988b).

Chemical Samples. Nepetalactone (I) was obtained form the catmint, *Nepeta cataria*, and nepetalactol (II) by reduction of I (Dawson et al., 1989b).

Gas-Chromatography–Mass Spectromety (GC-MS). GC employed an on-column injector and a 50-m × 0.32-mm-ID fused silica column with a cross-linked methylsilicone bonded phase (HP-1), 0.52 μm film thickness, linked directly to a 70–250 mass spectrometer and integrated data system (VG-Analytical). Conditions: GC, 30°C 5 min, 5°/min to 180°C isothermal; MS, electron impact at 70 eV, 230°.

RESULTS AND DISCUSSION

Leg extracts of oviparous females from three species of legume aphids showed the presence of the nepetalactone (I) and the nepetalactol (II), in differing proportions: *M. viciae*, approximately equal amounts (Dawson et al., 1987b); *A. pisum*, 1:25; *A. fabae*, 5:1 (Figure 1). Extracts from hind tibiae of *M. persicae* and *S. graminum* did not contain detectable amounts of either compound. However, a sample of volatiles entrained from calling females of *S. graminum* again showed the presence of both the lactone and the lactol, in the ration of 1:8, but little was obtained from noncalling females (Figure 2). This agreed with the earlier study of Eisenbach and Mittler (1980). Analysis of entrained volatiles from *M. persicae* by GC-MS using ion fragmentation techniques located the lactone (M,166) and the lactol (M,168) (Figure 3A–C) at the expected retention times. Although only trace amounts were present, spectra obtained by data enhancement using the mass spectrometer data system (Figure 3D) were satisfactory when compared with spectra from the authentic compounds (Pickett, 1990).

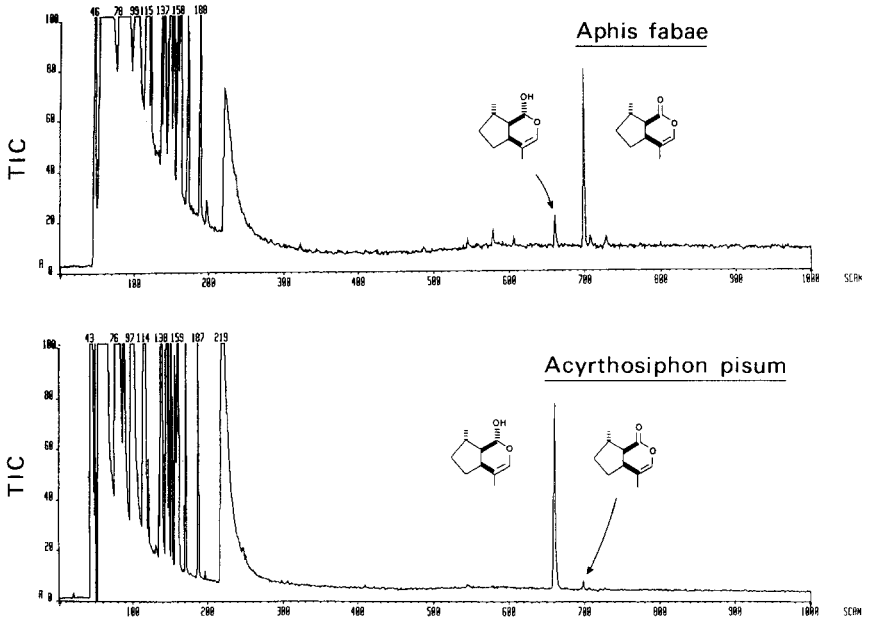


FIG. 1. GC-MS of leg extracts from oviparous *A. fabae* and *A. pisum*.

When similar air-entrainment techniques were applied to the three species of legume aphids, the ratio of I and II were very different from those found in the extracts (Table 1). In addition, while only 1–2 ng/aphid of lactone was present in leg extracts of *M. viciae* (Dawson et al., 1987), the amount produced by calling females can exceed 200 ng/day. This suggests that during calling, there is a continuous production of the lactol, probably from a glycoside precursor, and that this is then selectively oxidized to the lactone. A similar situation exists for the honeybee Nasonov gland pheromone (Pickett et al., 1981). In some instances, this may be to produce the correct pheromone ratio, but for *S. graminum*, it may provide a "switch-off" mechanism, as the lactone shows some inhibitory activity. Electrophysiological studies have shown that the sex pheromone is detected by the secondary rhinaria on the male antenna (Dawson et al., 1987b) and single-cell recordings demonstrated the presence of two types of olfactory cell in each rhinarium (e.g., Figure 4). In all the species so far investigated, the smaller amplitude cell responds to the lactone and the larger to the lactol. The clear discrimination in response is maintained even at high stimulus concentrations (Figure 5). The difference in impulse frequency elicited from the two cell types by these compounds is not yet understood.

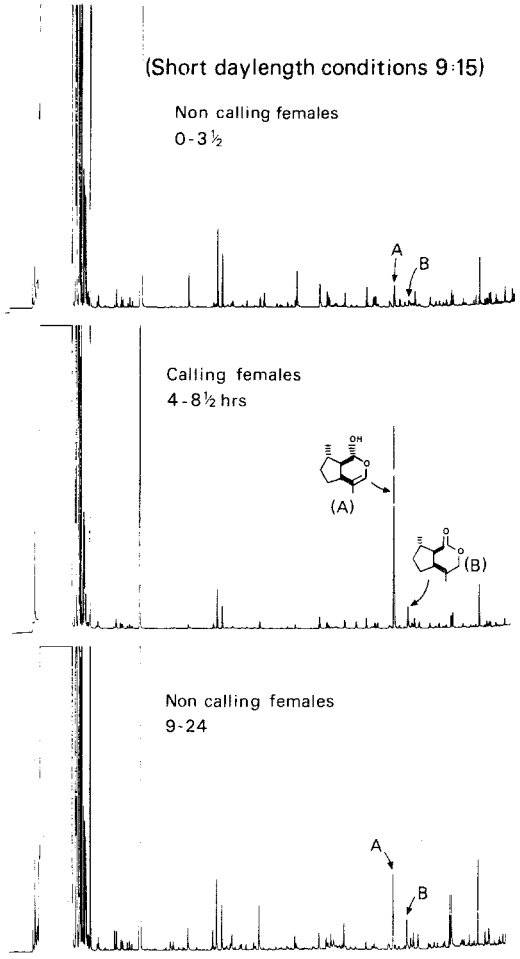


FIG. 2. Air entrainment extracts of volatiles produced by calling and noncalling *S. graminum* oviparae.

The ubiquity of the sex pheromone components may give rise to interspecific mating behavior. In a laboratory assay, males of *M. viciae* and *S. graminum* responded preferentially to conspecific oviparae but also exhibited considerable interest in other females. The level of response correlated directly with the ratios of lactone to lactol produced. Thus, high proportions of the lactone elicited strong responses form *M. viciae* but caused inhibition is *S. graminum* (Table 2). Further studies are in progress to confirm this hypothesis.

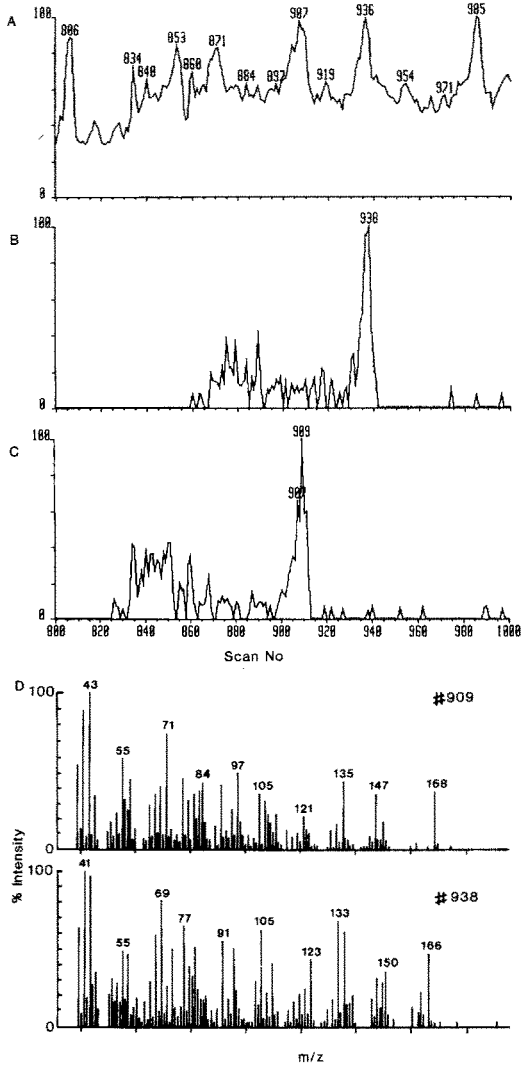


FIG. 3. GC-MS of *M. persicae* oviparae air entrainment volatiles. Total ion current (A). Ion chromatograms: *m/z* 166 (B) and *m/z* 168 (C). Mass spectra of the peaks corresponding to scan numbers 909 and 938 (D).

The role of the aphid sex pheromone is unclear, but it has been shown to have attractant effects over a short range: in a laboratory assay, 55% of males released in the center of the olfactometer moved directly towards the chamber containing oviparae. Since long-range attraction has not been demonstrated, it was considered unlikely that the sex pheromone would be useful in surveys or

TABLE 1. RATIOS OF NEPETALACTONE AND NEPETALACTOL IN LEG AND AIR-ENTRAINMENT EXTRACTS OF OVIPAROUS FEMALES

	Ratio lactone–lactol	
	Leg extract	Air-entrainment extract
M. viciae	1:1	6:1
A. pisum	1:25	1:1
A. fabae	5:1	29:1

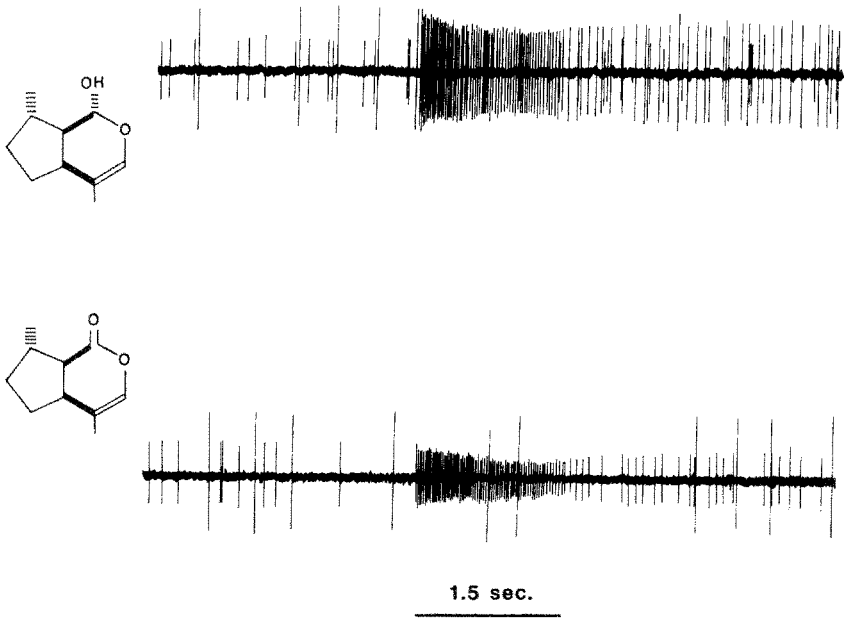


FIG. 4. Responses of two cells in a secondary rhinarium of an *A. pisum* male to stimulation with 100 ng of nepetalactol and nepetalactone.

mass trapping (Nault and Montgomery, 1977). However, the sex pheromone of the hop aphid, *Phorodon humuli*, which has recently been identified, is now proving to be extremely effective in attracting males to traps in hop gardens, although there is clearly an involvement of primary host-plant chemical cues. These results, when complete, will be published elsewhere. For some species, the sex pheromone shows considerable potential for monitoring aphids during their overwintering stage and for modifying male behavior in order to reduce the population.

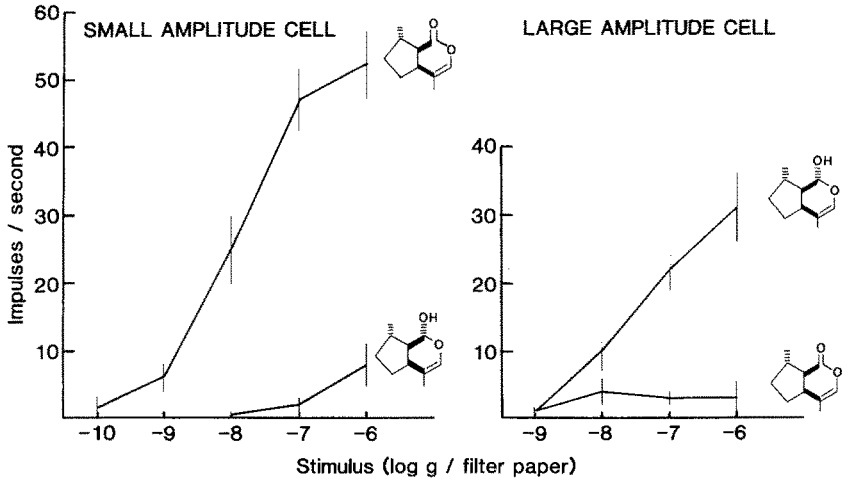


FIG. 5. Dose–response curves of male *M. viciae* olfactory cells to stimulation with nepetalactone and nepetalactol. Each point is a mean of three preparations.

TABLE 2. RESPONSES OF MALE APHIDS IN OLFACTOMETER TO OVIPARAE OF OTHER SPECIES: CUMULATIVE COUNTS OVER 15 MINUTES

A. *Megoura viciae* males.

B. *Schizaphis graminum* males.

Female species	Ratio of lactone–lactol	Mean no. males observed: In treated arm	Mean no. males observed: In each untreated arm	*P*
A.				
M. viciae	6:1	49	3	<0.001
A. pisum	1:1	48	5	<0.001
A. fabae	29:1	42	2	<0.001
S. graminum	1:8	21	11	<0.01
M. persicae	1:1.5	17	11	<0.05
M.v. asexuals		12	16	NS[a]
B.				
S. graminum	1:8	34	8	<0.001
A. pisum	1:1	28	9	<0.001
M. viciae	6:1	23	11	<0.01
M. persicae	1:1.5	21	11	<0.05
A. fabae	29:1	16	14	NS
S.g. asexuals		13	16	NS

[a] NS = not significantly different at probability level 0.05 (Student's *t* test).

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PRESENT AND FUTURE USE OF SEMIOCHEMICALS IN PEST MANAGEMENT OF BARK BEETLES [1]

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Abstract—Attractive compounds affecting the mass aggregation of bark beetle populations on host trees suitable for colonization usually consist of two obligatory components that act synergistically and species-specifically. Semiochemicals inhibiting response act on their own and seem less specific. From nearly 100 species investigated so far, mass aggregation can be simulated with commercial synthetics in about nine species of economic importance. Aspects leading to the application of attractants in monitoring and mass trapping pest populations affecting European spruce forests result from intensive coordinated research at the university, industry, and forestry level. Technology transfer was facilitated by, and adapted to, the infrastructure of European forestry; traps economically replace the trap tree methods conventionally used for centuries. Expected applications in the near future are refined monitoring methods to measure population levels and predict damages. Also, mass trapping should remain a worthwhile tool in preventing beetle damage in forests under management intensive enough to remove excessive breeding material. In the long run, response-inhibiting semiochemicals resulting in the dispersal of pest populations (*Ablenkstoffe*) may gain wider application. The spruce engraver *Ips typographus* L. and its associate *Pityogenes chalcographus* L. are used as examples to describe the feasibility of developing and applying inhibitors as new tools in the management of bark beetle pests: Applying a slow-release verbenone formulation (verbenone strip) wrapped around the trunk of spruce trees at breast height appears to protect spruces from destructive attack by *Ips typographus*, while small polyethylene ampullae containing terpinene-4-ol counteract aggregation of P. chalcographus. Inhibitors appear applicable in both strategies, damage prevention as well as damage restriction, and consequently may accommodate also pest control in less intensively managed forests. Future application of semiochemicals in the management of bark beetle pests will rest with the availability of effective means and

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methods and their acceptance by the forestry interest. This acceptance is presently somewhat hampered by misconceptions about mass trapping, and by (1) "missing links" in the knowledge of the beetles' dispersal and aggregation behavior, (2) the chemosynthesis of chiral pheromone components at the industrial level, and (3) legal barriers.

Key Words—Aggregation pheromones, inhibitors, *Ips typographus*, mass trapping, Pityogenes chalcographus, terpinene-4-ol, verbenone, Coleoptera, Scolytidae.

INTRODUCTION

The semisocial behavior by which populations disperse, aggregate, and shift attack makes bark beetles pests particularly vulnerable to biotechnical manipulation; both sexes respond to chemical cues generated initially by host-selecting "pioneer" beetles and, subsequently, by the aggregated population. As colonization of a host tree goes on, repulsive signals shift or terminate aggregation, and attraction simply ceases regardless of the source of the signals be it host, insect, or both.

Attractants. Exploiting the phenomenon of mass aggregation empirically, for centuries European foresters have monitored and controlled beetle infestations using trap trees. Today, we know that attractive compounds effecting aggregation en masse usually consist of at least two obligatory components that act synergistically. The synergism is somewhat species-specific while the components alone are not. From nearly 100 species investigated, aggregation can be simulated with synthetics only in about a dozen species, and, for at least nine of these, attractants are commercially available.

Ten years of experience with monitoring and mass trapping bark and ambrosia beetles using semiochemicals (see Bakke and Lie, 1989) are well documented and have led to the development of efficient trap technologies as an integrated part of control programs. Obviously, strategies for controlling pest populations using attractants recognize basic facts about bark beetle behavior and population dynamics, especially that (1) attractants concentrate the flying population near baited traps, but only a portion of the aggregating beetles are caught by the traps: and (2) trapping does reduce the local pest population; however, it has little influence on the population dynamics of the beetles, which is dictated by the absence or presence of suitable breeding material.

Trapping, therefore, is only efficient when integrated into an intensive management system that affords the prompt removal of obviously suitable host material such as logging debris and "slash-on-the-stump" from the forest. This requires discrete forest units with accessibility and continuous surveillance, i.e., an infrastructure that still exists in European countries where traps now economically replace the conventional trap tree method.

While the various commercially available traps are excellent tools in monitoring bark and ambrosia beetle populations alike, both monitoring and mass trapping in Europe are directed primarily against aggressive bark beetles such as *Ips typographus* and *Pityogenes chalcographus*, to predict and prevent damage (see Weslien et al., 1989). Control trapping should be restricted to mass use of pheromone traps along with sanitation measures in selected areas that are sensitive to infestations. Scattered trap deployment throughout host-type forests generally does not provide control, rather it tends to stimulate scattered infestations of trees near the traps.

Without doubt, at present, the use of semiochemicals focuses on the application of attractants linked to intensive management. However, in the long run, response inhibiting semiochemicals effecting the dispersal of pest populations may gain wider use since they do not require the labor involved in deploying and maintaining traps and are applicable in a more extensive forest management.

Inhibitors. Semiochemicals that interfere with aggregation and/or induce dispersal are variously termed inhibitors, disruptants, repellents, or antiaggregants. Search for candidate compounds may turn to various sources: (1) antipodes of attractive compounds, occasionally called nonnatural enantiomers, such as (*R*)-*cis*-verbenol (Vité et al., 1976), (*S*)-(−)-ipsenol (Birch et al., 1977), (*S*)-(+)-ipsdienol (Vité et al., 1978) or (−)-*endo*-brevicomin (Vité et al., 1985); (2) ketones of known attractant alcohols such as 3-methyl-2-cyclohexen-1-one (Kinzer et al., 1971; Vité and Pitman, 1972) or verbenone (Renwick and Vité, 1969); (3) Pheromones of sympatric or competing species (allomones) such as (*S*)-(−)-ipsenol attracting *Pityokteines curvidens* L. but repelling *Ips typographus*; (4) volatiles of nonhost plants or host trees such as ethanol, which indicate unsuitable breeding material (König et al., 1981).

Inhibitors may be group-specific rather than species-specific and act on their own; there is no evidence of synergistically acting inhibitor components. While the industrial development of controlled release dispensers is well advanced, our interest concentrates at present on two basic questions: (1) Which compounds should be formulated for use in suppressing mass attack of bark beetles in Norway spruce [*Picea abies* (L.) Karst.] stands, i.e., *Ips typographus* L. and *Pityogenes chalcographus* L.? (2) What type of application is most effective in preventing trees from attack by bark beetles?

METHODS AND MATERIALS

Field studies were performed from 1986 through 1989 in 60- to 80-year-old stands of Norway spruce in the Upper Rhine Valley and Black Forest Mountains near Freiburg, F.R.G.; endemic populations of *Ips typographus* and *P.*

chalcographus were present. The materials used were: (*S*)-verbenone, Bedoukian, Danbury, Connecticut, 97% (64 *ee*); (*S*)-cis-verbenol, Bedoukian, Danbury, Connecticut, 96%; (+)-terpinen-4-ol, Bedoukian, Danbury, Connecticut, 92% (20 *ee*); methyl-(2*E*, 2*Z*)-2, 4-decadienoate, Bedoukian, Danbury, Connecticut; rac. ipsenol, Borregaard, Sarpsborg, N. 97%; (*S*)-(−)-ipsenol, Ciba-Geigy, Basel, Switzerland; 2-methyl-3-buten-2-ol, Aldrich, Steinheim, F.R.G. 97%.

Standard baits were Pheroprax (*I. typographus*) and Chalcoprax (*P. chalcographus*), both commercial products provided by Shell-Agrar, Ingelheim/Rhein, F.R.G.

If not noted otherwise, the materials were dispensed as: (1) PE dispenser: an envelope made from 0.05-mm, low-density polyethylene 12 × 7 cm, containing a 5 × 5-cm cellulose pad to absorb the test liquid in 2- or 4-ml amounts; (2) PE ampullae: a rigid two-chamber, horseshoe-shaped polyethylene container provided by BASF, Ludwigshafen, F.R.G., each chamber with the capacity of 2 ml; (3) verbenone strip: a flexible black plastic band; 2 cm in width provided by BASF, Ludwigshafen, F.R.G., containing 10 g verbenone per meter.

Traps used were either flight barriers (Theysohn *Schlitzfallen* and Röchling *Flachtrichter*) hung from strings fastened to trees, or commercial units of three barriers fixed to a metal post (Theysohn *Dreifallenstern*).

RESULTS

As indicated by previous studies (Baader and Vité, 1986) methyl-(2*E*, 4*Z*)-2, 4-decadienoate (*E*, *Z*-MD) reduced the response of *Ips typographus* to traps baited with Pheroprax by 40% (Table 1). However, as reported earlier by Bakke (1981, 1987), (*S*)-verbenone proved consistently superior in the reduction of trap catches. Combining (*S*)-verbenone with *E*,*Z*-MD and additional compounds known to lower trap catches, i.e., ethanol (König et al., 1981) and

TABLE 1. RESPONSE OF *Ips typographus* TO RÖCHLING-SCHLITZFALLEN BAITED WITH PE DISPENSER CONTAINING 4 ml *E*,*Z*-MD AND/OR PHEROPRAX[a]

Bait	Average number (and range) of beetles responding	Reduction (%)
Pheroprax	39.3 (9–116)	
Pheroprax and *E*,*Z*-MD	23.1 (3–53)	41.2

[a] Wittental May 14–26, 1986; 16 replications.

TABLE 2. RESPONSE OF *Ips typographus* TO TRAPS SCHLITZFALLEN BAITED WITH (*S*)-VERBENONE, A MIXTURE OF RESPONSE INHIBITING SEMICHEMICALS, AND/OR PHEROPRAX[a]

	X^b	SE	Reduction (%)
Standard[c]	37.9a	8.44	
Standard and (*S*)-verbenone[d]	6.9b	1.21	81.8
Standard and mixture[e]	14.5b	2.85	61.7

[a] Buchenbach, Black Forest Mts., June 9–July 17, 1989, 35 replications.
[b] Column means followed by different letters are significantly different; Kruskal–Wallis H test ($P = 0.0011$) and two-tailed Mann–Whitney U test ($P \leq 5\%$).
[c] PE dispenser 2 ml; 2-methyl-3-buten-2-ol and (*S*)-*cis*-verbenol, 20 : 1.
[d] PE dispenser containing 4 ml (*S*)-verbenone.
[e] PE dispenser containing 4 ml of (*S*)-verbenone, *E*,*Z*-MD, ipsenol, and ethanol, 1 : 1 : 1 : 1.

ipsenol (Bakke, 1981) at equal amount, did not result in significantly greater trap catch reduction (Table 2). Response inhibition effected by ipsenol alone appeared to be due to the (−)-enantiomer, the aggregation pheromone of *Pityokteines curvidens* L. (Harring, 1978), a sympatric bark beetle pest of white fir, *Abies alba* Mill.

Terpinen-4-ol efficiently suppressed *P. chalcographus* response to traps baited with Chalcoprax (Table 3); the closer the release to the trap, the stronger the reduction (Figure 1). Furthermore, the male-specific compound 1-hexanol (Francke et al., 1977) and monoterpenes such as (−)-limonene may interfere with aggregation but not as much as terpinen-4-ol (Table 4). Verbenone affected *P. chalcographus* aggregation little; terpinen-4-ol had little effect on *Ips typographus* aggregation (E. Baader and J.P. Vité, unpublished data).

The BASF No. 5 ampullae proved superior for slow release of terpinen-4-ol compared to PE dispensers (Table 5). The BASF verbenone strip reduced

TABLE 3. RESPONSE OF *Pityogenes chalcographus* TO RÖCHLING-FLACHTRICHTER BAITED WITH 4 ml TERPINEN-4-OL IN PE DISPENSER AND/OR CHALCOPRAX[a]

	P. chalcographus		Reduction
Bait	X^b	+SE	(%)
Chalcoprax	481.5	+190.51	
Chalcoprax plus terpinen-4-ol	65.8	+14.52	82.3

[a] Freiburg-Kappel, April 29–July 3, 1987.
[b] The mean values differ significantly with $P \leq 5\%$, ANOVA ($F = 0.0431$).

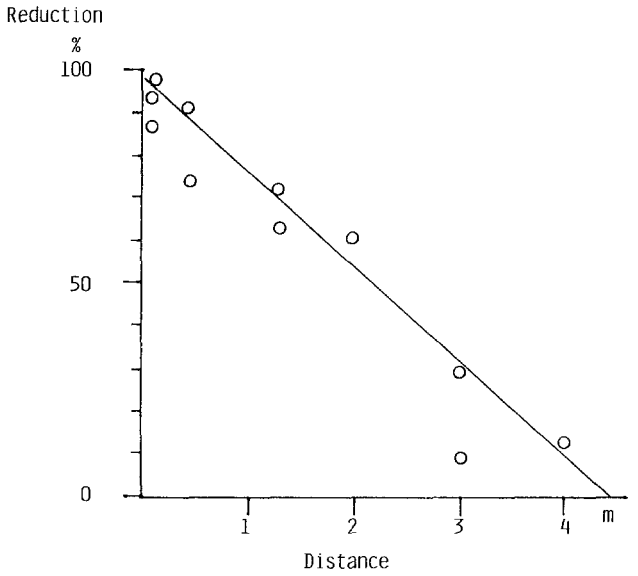


FIG. 1. P. chalcographus: reduction in response to a Theysohn-fallenstern baited with Chalcoprax and terpinene-4-ol. Six PE dispensers containing 4 ml terpinen-4-ol each were deployed in a circle at various distances around the trap center. Mooswald, Upper Rhine Valley, May 1989.

aggregation of *Ips typographus* most effectively when wrapped around the trunk of Norway spruce at breast height (Figure 2).

Deployment of the pheromone strips influenced their effectiveness. Verbenone strips hung freely from strings around a Pheroprax-baited trap lost effectiveness at a short distance away from the trap but were still effective at similar distances when affixed to the trunk of trees. For example, placing a 25-m-long verbenone strip at breast height and in a straight line throughout a stand of Norway spruce reduced the catch in traps on either side by approximately 66%;

TABLE 4. RESPONSE OF *Pityogenes chalcographus* TO RÖCHLING-FLACHTRICHTER BAITED WITH 4 ml (−)-LIMONENE IN PE DISPENSER AND/OR CHALCOPRAX[a]

Bait	*P. chalcographus* X^b	+SE	Reduction (%)
Chalcoprax	499.5	+108.14	
Chalcoprax plus (−)-limonene	221.8	+68.02	55.6

[a]Hinterzarten, Black Forest Mts., May 29–July 3, 1987.
[b]The mean values differ significantly with $P \leq 5\%$, Kruskal–Wallis H test ($P = 0.0020$).

TABLE 5. RESPONSE OF *Pityogenes chalcographus* TO THEYSOHN-SCHLITZFALLEN BAITED WITH 4 ml TERPINEN-5-OL IN PE DISPENSER OR PE AMPULLAE, AND/OR CHALCOPRAX[a]

Bait	*P. chalcographus* X^b	*P. chalcographus* SE	Reduction (%)
Chalcoprax	171.5a	40.62	
Chalcoprax plus terpinen-4-ol/PE dispenser	37.9b	8.29	77.9
Chalcoprax plus terpinen-4-ol/PE ampullae	15.8	6.36	90.8

[a]Buchenbach, Black Forest Mts., May 8–26, 1989, 24 replications.
[b]Column means followed by different letters are significantly different; Kruskal–Wallis H test ($P = 0.0000$) and two-tailed Mann–Whitney U test ($P \leq 5\%$).

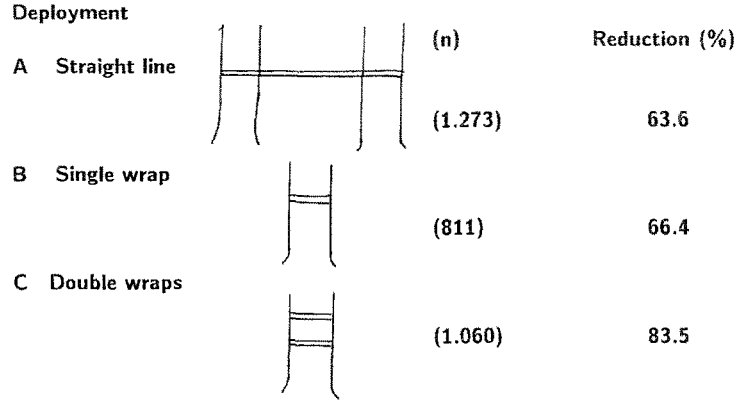


FIG. 2. Application of verbenone strip: influence of deployment on the reduction of *Ips typographus* caught in eight Pheroprax-baited Röchling traps place in the test area compared to eight traps in the untreated control area.

the same result was obtained by wrapping half the amount of the strip around trees along this line. Using the full load of the strip on the trees, the reduction rose to 84%.

DISCUSSION

Forest pest management strategies are strongly governed by the infrastructure of the forest interests. Only under an intensive forest management system does mass trapping of bark beetles appear feasible as an integrated part of dam-

age prevention in the classical strategy of *saubere Wirtschaft*. Under an extensive forest management system the strategy is damage restriction, and mass trapping appears of little use where abundance of suitable host material provides the basis for continuous bark beetle proliferation. Here, the application of inhibitors that counteract aggregation appears the better technique in curbing damage locally (Payne and Billings, 1989). Nevertheless, in both systems bark beetle traps serve very well for monitoring, as was recently shown for *Ips typographus* (Weslien et al., 1989) and has been used for years by various Central European forest services (Vité, 1990).

However, the application of inhibitors has predictable limitations. Inhibitors may reduce but not prevent successful attack on cut (Bakke, 1987) or windthrown timber. On the other hand, the prevention of fatal attack, possibly by reduction and/or delay of attacks on standing timber, may suffice to mobilize tree resistance and render the attack unsuccessful. We, therefore, concentrated our work on how to protect standing Norway spruce from successful attack by *Ips typographus* and *P. chalcographus* using inhibitors. Among the many semiochemicals counteracting aggregation by *Ips typographus*, (*S*)-verbenone appears the most efficient so far. Hypothetically, verbenone interrupts the response to *cis*-verbenol, the major component of the contact pheromone for the species. However, the actual role of verbenone, as well as ipsdienol and ipsenol in the aggregation process, remains open to speculation. The detection of ipsenol by male *Ips typographus* may suggest a density regulation mechanism (Schlyter et al., 1987) since racemic ipsenol inhibits aggregation response at higher concentrations. However, natural ipsenol is unlikely to be the (−)-enantiomer, which attracts *Pityokteines curvidens* (Harring, 1978) but inhibits *Ips typographus* response (Bakke, 1981). In our model (Figure 3), *Ips typographus* uses (*R*)-(−)-ipsdienol (Francke et al., 1980) and (*R*)-(+)-ipsenol as frass pheromones in the colonization of temporary habitats where host conditions minimize verbenol production (Kohnle, 1984).

The verbenone actually released by *Ips typographus* appears minute in comparison to the verbenone released by competitors such as *Pityogenes chalcographus* (Francke et al., 1977) and *Ips amitinus* Eichh. (Francke et al., 1980). A similar situation is found with *P. chalcographus*, which releases trace amounts of terpinene-4-ol during colonization while its competitor *Polygraphus poligraphus* L. uses the same compound as an attractive principle (Kohnle et al., 1985). Both compounds, (*S*)-verbenone and terpinen-4-ol have the advantage of commercial availability and easy application on trees, i.e., terpinen-4-ol in ampullae and verbenone as a polyethylene strip. However, we are unable to explain why these formulations attached to trees work better than other forms of application, except for the rapid transformations of the ultraviolet-degradable verbenone into inactive chrysanthenone when deployed as PE granules (Vité, 1990).

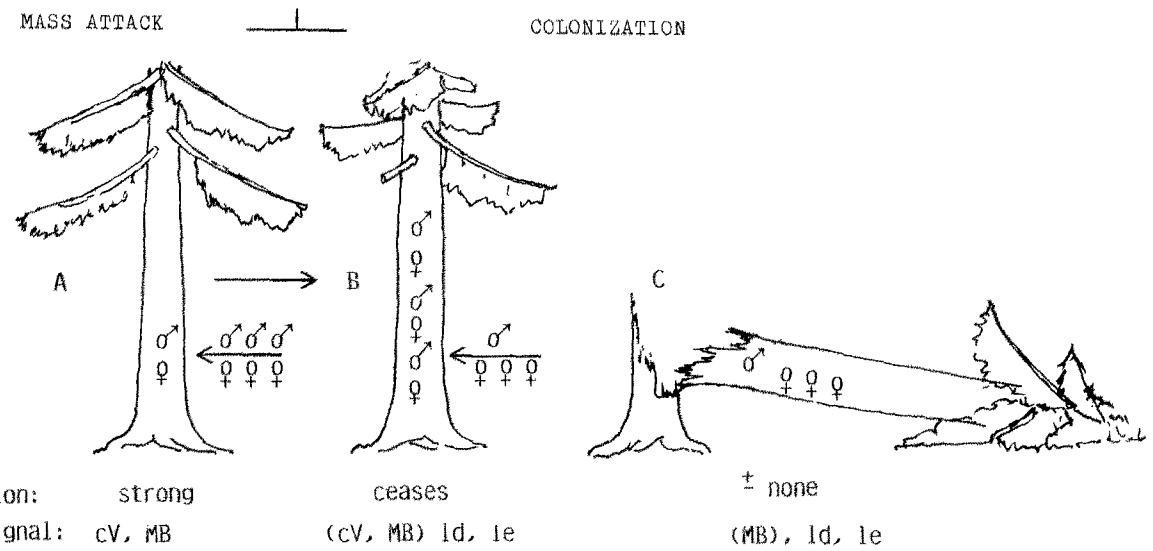


FIG. 3. Conceptual model of alternative pheromone systems governing secondary attraction of *Ips typographus* to host trees resistant (A, B), and susceptible (C) to colonization. Upon contact with the host's oleoresin, (*S*)-*cis*-verbenol (cV) and 2-methyl-3-butene-2-ol (MB) synergize mass attack by both sexes; upon feeding (B), male specific (*R*)-(−)-ipsdienol (Id) and (*R*)-(+)-ipsenol (Ie) signal successful colonization attracting preferably female beetles; in the absence of resin exudation, the "frass" pheromones Id and Ie mark habitats temporarily suitable for breeding.

Our present knowledge of the aggregation and dispersal behavior of bark beetles in flight remains lacking. Particularly with regard to the interaction between host type and area attractiveness, visual stimuli, and secondary attraction by aggregation pheromones. As with *Ips typographus*, it is well documented that trap catches decrease with distance from the host stand (Vaupel et al., 1986) and that freshly cut areas favor higher beetle activity than older clearings or other open areas (Austara et al., 1986). From our results we must conclude that the host tree "image" is an essential factor to be considered in efficient manipulation of *Ips typographus* with semiochemicals, attractants, as well as inhibitors.

Also lacking is our knowledge of how inhibitors or repellents act in the reduction of trap catches. Usually, the threshold for perception of inhibitors is somewhat higher than for attractants, and inhibitors seem to be effective only in specific concentrations. Experimentally, reduction in response is highest when attractants and inhibitors are placed in the same position so that both odor plumes become congruent, but the question remains why unrelated compounds such as ethanol and dipentene, or verbenone and ipsenols are effective on their own but do not increase the disruption effects when combined.

Besides the "missing links" in our knowledge of pheromone perception and beetle behavior that lead to attraction or inhibition, technology transfer is hampered by some legal barriers such as EPA requirements (Punja, 1989) and the lack of enantioselective chemosynthesis of attractants and inhibitors at the industrial level. Obviously, forest pest management is a poor and uncertain market for the industry to develop and invest in under the present conditions and public opinion (Wulf, 1987). However, as the European experience shows, this obstacle may be overcome by combined efforts at the university, industry, and forestry levels.

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HOST-MARKING PHEROMONES IN LEPIDOPTERA, WITH SPECIAL REFERENCE TO TWO *Pieris* SPP.

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Abstract—Adult females of several lepidopteran species avoid oviposition near conspecific eggs. Laboratory and field studies indicate that visual factors as well as chemical host markers may be involved in egg avoidance behavior. The oviposition-deterring pheromones (ODP) of two pierids, *P. brassicae* and *P. rapae* have been studied in considerable detail. The ODPs are probably produced in the female accessory glands and contain volatile and nonvolatile components. They are perceived by nonspecialized antennal or tarsal receptors, respectively. The ODP of *P. brassicae* affects oviposition of *P. rapae* females and vice versa. ODPs show promise for insect control programs, but more information on oviposition behavior in the presence of ODP under field conditions is required.

Key Words—oviposition-deterring pheromone, *Pieris brassicae*, *Pieris rapae*, oviposition, Lepidoptera, Pieridae, dispersal behavior, pheromone perception, oviposition-deterring pheromone receptors, electroantennogram.

INTRODUCTION

Nature is not a Garden of Eden, but an abode of many competing forms of life. Even sibling insects would have to compete for nourishment were it not that the solicitude of their mother reduces this hazard. To this end, several insect species produce a host-marking substance concomitantly with egg deposition (Roitberg and Prokopy, 1987). This chemical signal may prevent a female returning to the same site, or conspecifics ovipositing near previously laid eggs. Field and laboratory studies have shown the use of host-marking pheromones in lepidopterans (Table 1), dipterans, coleopterans, and hymenopterans (Prokopy, 1981).

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TABLE 1. CASES OF EGG AVOIDANCE BEHAVIOR IN LEPIDOPTERANS

Species	Place of study	Stimulus type		Reference
		Chemical	Visual	
PAPILIONIDAE				
Battus philenor	field			Rausher, 1979
PIERIDAE				
Pieris brassicae	lab	+	+	Rothschild and Schoonhoven, 1977; Klijnstra, 1985
	field			Kirby and Spence, 1863
Pieris rapae	lab	+		Rothschild and Schoonhoven, 1977
	field			Kobayashi, 1965
Pieris sisymbrii	field		+	Shapiro, 1981; Kellogg, 1985
Anthocharis sara	field		(+)	Shapiro, 1980
A. cardamine	field			Wiklund and Åhrberg, 1978; Thomas, 1984
NYMPHALIDAE				
Danaus plexippus	field/lab			Urquhart, 1960; Dixon et al., 1978
Heliconius	field		+	Gilbert, 1975; Williams and Gilbert, 1981
NOCTUIDAE				
Heliothis zea	field			Scott, 1977
Hadena bicruris	field	(+)		Brantjes, 1976

Since butterflies have generally well-developed eyes, egg avoidance may be accomplished by visual cues, especially when the eggs are brightly colored. Thus the females of *Pieris brassicae*, *Heliconius* spp., and several other species refrain from egg laying not only near eggs of conspecifics, but also near artificial or natural egg mimics or eggs from heterospecific females (Rothschild and Schoonhoven, 1977; Gilbert, 1975; Shapiro, 1980; 1981). In this paper only chemical cues will be discussed.

In the case of *P. brassicae* and *P. rapae*, it was found that cabbage leaves from which eggs were carefully removed, remained less acceptable for oviposition than "clean" control leaves, indicating that some chemical associated with egg laying was left, which may deter other females from ovipositing (Rothschild and Schoonhoven, 1977). In addition to a nonvolatile component, the host marker probably also contains volatiles.

NONVOLATILE COMPONENT OF OVIPOSITION-DETERRENT PHEROMONE

A compound that acts as an oviposition-deterrent pheromone (ODP) can be extracted by washing eggs with water (Behan and Schoonhoven, 1978) or methanol (Klijnstra, 1986). The egg wash can be tested for its ODP activity by spraying it on a cabbage leaf and then offering it to a gravid female, together with a control leaf sprayed with solvent only. By varying the concentration of the egg wash, a dose–response curve can be determined (Figure 1). Weak but significant responses to leaves treated with 1 ml egg wash obtained from four eggs were observed. Strong responses with deterrence levels of 80–95% require washes from 500 or more eggs/ml.

Accessory Glands. To locate the source of the ODP, several parts of the gravid female's abdomen were screened for ODP activity. The accessory glands (Matsuda, 1976), which produce an adhesive (Hinton, 1981), appear to contain ODP, whereas ripe ova excised from the ovaria of a female butterfly are inactive. During egg deposition, the accessory gland secretion is used to attach the eggs to the oviposition site. When the eggs are carefully removed with a brush,

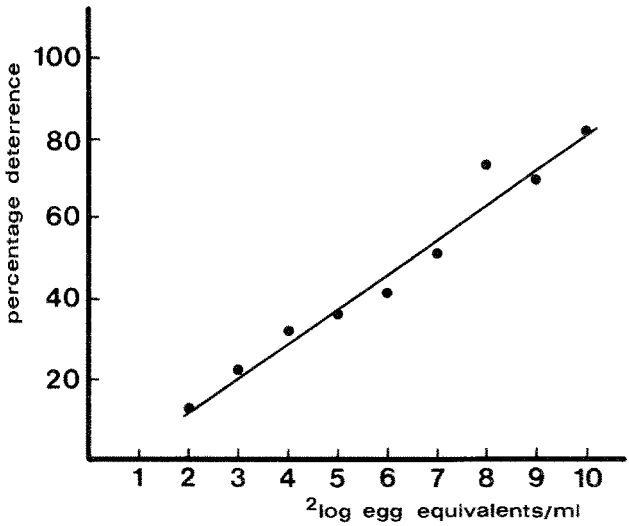


FIG. 1. Dose–response curve of oviposition avoidance behavior to methanol egg washes in females of *P. brassicae*. Linear regression of percentage deterrence and ^{2}log eggwash concentration (after Klijnstra, 1986). Percentage deterrence = (A − B) × 100/(A + B), where A and B represent the numbers of eggs laid on control and treated leaves, respectively.

apparently some of the behaviorally active principle remains on the leaf surface, since ovipositing females prefer clean leaves to leaves from which eggs have been removed. The ODP also is present on the egg chorion from where it is gradually released when rinsed (Behan and Schoonhoven, 1978; Schoonhoven et al., 1981; Klijnstra, 1986).

In *P. brassicae* the activity of two pairs of glands is somewhat lower than that of a wash from 500 eggs (Schoonhoven et al., 1990b). Since, during a 5-min wash, only a small fraction of the ODP is removed from the eggs (Schoonhoven et al., 1981), the amount of the ODP in the glands, as compared to the amounts present on the eggs, seems relatively small. Presumably, the glands do not store appreciable amounts of ODP, but produce it on demand. The fact that the glands of *P. rapae* show a much lower activity than those of *P. brassicae* agrees with this presumption, especially when one considers that *P. rapae* lays single eggs; in contrast, *P. brassicae* oviposits in clusters of 20–100.

Chemoreception of ODP. Many lepidopterans use tarsal receptors for chemical assessment of leaf surfaces when selecting an oviposition site. Contact chemosensory hairs contain several neurons that may respond to oviposition stimulants, feeding stimulants such as sugars, or oviposition deterrents. In *P. brassicae* and *P. rapae*, one of the neurons is strongly stimulated by egg washes and extracts of accessory glands (Behan and Schoonhoven, 1978; Klijnstra and Roessingh, 1986; Schoonhoven et al., 1990a). A strophanthidin glycoside, which inhibits oviposition in *P. brassicae*, also stimulates the ODP-sensitive neuron (Rothschild et al., 1988). Apparently this neuron responds to a variety of substances, possibly differing in chemical structure, which act as oviposition deterrents. Tarsal receptors sensitive to water-soluble ODP also have been found in the apple maggot fly (Crnjar and Prokopy, 1982; Bowdan, 1984) and the European cherry fruit fly (Boller et al., 1987).

The ODP component in the egg wash is probably nonvolatile, since gravid females do not show differences in prealighting behavior to leaves treated with egg washes as compared to controls (Klijnstra, 1985). When kept under room conditions, dried egg washes on a glass surface retain activity for periods of at least seven weeks, also indicating that its volatility is low or absent (Schoonhoven et al., 1981).

The question arises whether ovipositing females, after landing on the upper surface of a cabbage leaf, can perceive the contact ODP present in the egg and the cement on the underside of the leaf. Butterflies are known to exert a very thorough chemical inspection of the upper leaf surface by extensively and repeatedly "drumming" the substrate with their foretarsi (Rothschild, 1987). It is difficult to understand how they could perceive the presence of an ODP under natural conditions, if there are no physicochemical mechanisms at the

leaf surface or transport routes through the leaf interior that enable some of the relatively large amounts of ODP stored in the eggs to spread over the upper leaf surface.

Generally, *Pieris* butterflies remain, after landing, on the upper side of the leaf during oviposition, and they may touch the underside of the leaves only with their ovipositors. However, there is no evidence for the presence of ODP receptors located on the female's abdomen or ovipositor (Klijnstra and Roessingh, 1986).

If the nonvolatile ODP is normally of no significance to the ovipositing female, natural selection should tend to eliminate its production, since the ODP serves as a kairomone to parasitoids (see below) and since production presumably costs something.

VOLATILE COMPONENTS OF OVIPOSITION-DETERRENT PHEROMONE

The observation that there is a reduction in the number of aerial visits (without leaf contacts) to plants and leaves that have been used for egg laying, independent of the presence or absence of eggs, indicates that airborne cues associated with oviposition are perceived from a distance (Rothschild and Schoonhoven, 1977).

The electroantennogram (EAG) technique may reveal whether or not the antennal olfactory system is stimulated by certain volatiles. Antennae of both *P. brassicae* and *P. rapae* females produce clear EAG responses when exposed to airstreams that had passed over conspecific eggs, whereas eggs of *Mamestra brassicae*, when used as a control, did not elicit a reaction (Schoonhoven et al., 1990b). No responses to (dried) egg washes were obtained. These results show that volatiles emanating from intact eggs stimulate antennal receptors. This could be verified by recording neural responses from individual olfactory cells. Egg odors appeared to stimulate certain so-called generalist receptors, indicating that the insects do not posses specialized "egg-odor" receptors (Behan and Schoonhoven, 1978; Otter et al., 1980). Although it remains to be shown unambiguously that these egg volatiles act as oviposition deterrents, it is interesting to note an analogy between the antennal and tarsal receptors, because the latter also seem to possess generalized deterrent receptors.

INTERSPECIFIC EFFECTS

The marker substance produced by one species (*P. brassicae* or *P. rapae*) deters the other species from laying eggs, thus potentially reducing interspecific competition for similar food resources in the same habitat (Schoonhoven et al.,

1990b). Whether or not the ODPs of the two pierids are chemically identical remains to be analyzed. In either case, it seems likely that both species profit from the fact that they can perceive each other's, which thus should be classified as synomones. Synomonal effects of epideictic pheromones also have been demonstrated in some closely related fruit fly species, which may partly or fully recognize the presence of a heterospecific ODP (Prokopy et al., 1976).

Several examples are known of host markers produced by insects, which are utilized by their natural enemies as host-finding cues (Prokopy, 1981). Egg washes of *P. brassicae* have a kairomonal effect on its egg parasite *Trichogramma evanescens* (Noldus and van Lenteren, 1985), although another *Trichogramma* species is deterred (Pak and de Jong, 1987). The accessory glands of two other lepidopterans, *Spodoptera frugiperda* and *Heliothis zea*, contain kairomones that influence the behavior of some of their hymenopterous parasitoids. High-molecular protein fractions isolated from the gland material induce behavioral responses in these parasitoids (Nordlund et al., 1987).

USE OF ODPs IN PEST CONTROL

In a large field cage with full-grown cabbage plants, most of them sprayed with egg wash, but some of them unsprayed (controls or "trap" plants), ovipositing *P. brassicae* females were observed not to discriminate between treated and untreated plants. However, after contacting sprayed plants, their preoviposition flights lasted longer than in control experiments in which none of the plants was sprayed, and the females visited several more plants before starting to oviposit. Apparently, after contacting ODPs, the insects show an increased dispersal activity (Klijnstra and Schoonhoven, 1987). Moreover, in laboratory experiments, females, after touching a "clean" leaf, showed a higher tendency to stay around or return to this leaf than in the case of an ODP-treated leaf (Klijnstra, 1985). This observation agrees with the assumption that dispersal movements are stimulated by tarsal contacts with ODP. Similar results have been reported for fruit flies, which were found to abandon rapidly trees carrying fruits marked with ODP (Roitberg et al., 1982).

ODPs, as natural and species-specific compounds that inhibit oviposition, seem ideal chemicals to reduce crop infestation by insects utilizing them, since they probably produce very low environmental risks. When assessing the potential role of lepidopteran ODPs in pest control programs, the following points need to be taken into account:

1. Even at relatively high concentrations, the ODP does not show absolute deterrence if the second clutch on a plant has an expected survival chance greater than zero (van Alphen and Visser, 1990). Moreover, egg avoidance behavior

in the natural situation is, in *P. brassicae*, partly based on visual stimuli (Rothschild and Schoonhoven, 1977).

2. In *P. rapae*, other factors than egg avoidance behavior may, under natural conditions, dominate the choice of an oviposition site, suggesting that ODP may be of only limited value in insect control programs. In several field studies (cited in Schoonhoven et al., 1990a), no evidence could be found for the existence of egg avoidance behavior in this species, although Kobayashi (1965) noted that eggs are laid more evenly and extensively with increased density.

3. *Pieris* butterflies only respond to ODPs when they have access to ODP-free host plants. Therefore, ODP sprayings of a cabbage crop would probably only be effective when the edges of the field remain unsprayed, and thus serve as trap plants. The natural tendency of pierids to oviposit along edges, rather than in the interior of stands (e.g., Jones, 1977; Wiklund and Åhrberg, 1978), would enhance the efficacy of this strategy.

4. Water-soluble ODPs may be washed off easily by rain and thus be of limited value in a wet climate. However, the spraying of an entire cherry tree with water-soluble ODP of the European cherry fruit fly during a wet summer yielded a 77% reduction in the number of infested fruits when compared to untreated control trees (Katsoyannos and Boller, 1976). This indicates that not all of the active substance is washed off the whole plant. Even after high-intensity natural rains, 39–50% of pheromone activity (in this case for the apple maggot fruit fly) remained (Averill and Prokopy, 1987). The fact that *Pieris* ODP is only gradually released from eggs may reflect some absorbance to waxy surfaces, such as those of eggs and plant leaves. This diminishes the chance that all of the activity will be lost after a rain shower.

5. The *Pieris* ODP seems to possess a high chemical stability (Schoonhoven et al., 1981; Klijnstra and Schoonhoven, 1987), which is considered a useful trait for application purposes. It would be interesting to quantify its stability under field conditions in view of Mangel and Roitberg's (1989) notion that ODPs should have a predictable half-life.

6. The fact that *Pieris* ODP modifies behavior of natural enemies should be taken into account when using ODPs for insect control. Further research is required to investigate whether or not under such circumstances the behavior of natural enemies is modulated by ODP in such a way that host-finding success is increased or reduced.

7. Several examples of successful use of behavior-modifying chemicals (Jutsum and Gordon, 1989) are an incentive for conducting more research on the potential usefulness of ODP. The finding that, under optimal conditions, cherry fruit fly infestation could be reduced by as much as 90% when applying its marking pheromone to whole cherry trees (Katsoyannos and Boller, 1980), also holds a promise for success in other insect groups.

The listed uncertainties and gaps in our knowledge of the detailed role of ODPs in the complex oviposition behavior will probably prevent its successful use in insect control within the next decade.

CONCLUSION

Host-marking pheromones constitute a chemical communication network that may affect conspecific and heterospecific herbivores, as well as some of their natural enemies. The intricacy of these networks is a marvel to the nature lover, a challenge to the biologist, and a possible new avenue to the applied entomologist.

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CHEMICALS INVOLVED IN HONEYBEE–SUNFLOWER RELATIONSHIP

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Abstract—We present a review of work on the plant chemicals involved in the honeybee–sunflower model system. Combined behavioral and chemical analyses were conducted under natural and controlled conditions. First the distribution of forager bees' visits on two pairs of sunflower genotypes producing a different level of hybrid seed yield was recorded under pollen-proof tunnels. Mirasol parental lines producing high seed yields were visited at random, whereas forager bees visited preferentially the female parental line of Marianne, resulting in low seed yield. Nectar samples collected on the genotypes were analyzed by gas chromatography. Fructose, glucose, and sucrose were identified. Parental lines of Mirasol showed similar sugar profiles, whereas the female line of Marianne contained higher amounts of sucrose than the male line. We assume that the bees' preferences between genotypes might rely on differences in the sugar composition of floral nectars, especially in the amount of sucrose. Aromas from headspace collection were compared between pairs and periodically during the flowering period. Of the 144 components indexed for Marianne lines and 136 components for Mirasol lines, 17 of the components for Marianne lines and 18 for Mirasol lines differed significantly according to flowering stage. Significant differences appeared in eight of the 134 components of Marianne lines and in 20 of the 250 components for Mirasol lines. Such differences, even restricted to a few components, might account for honeybees' discrimination between genotypes or flowering stage. Experiments then were conducted in a flight room using an artificial flower device. A total volatile extract was used as a conditioning scent previous to the test where the total extract was successively

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compared to several of its subfractions. Fractions significantly less visited than the total extract were discarded, whereas fractions confused with the total extract were kept. From step to step, a restricted fraction of 28 polar components, among which 15 were identified, was shown to be as active as the initial conditioning extract. These data emphasized honeybees' abilities to generalize from simplified to more complex chemical information. Finally, this work considers the possible use of such plant chemicals, from nectars or aromas, either as targets for genetic modification of crop plants or as direct attractants when sprayed on the crop, for the improvement of entomophilous cross pollination.

Key Words—Sunflower, *Helianthus annuus*, honeybee, *Apis mellifera*, Hymenoptera, Apidae, foraging behavior, aroma, nectar, plant chemicals, conditioning, olfactory discrimination, pollination.

INTRODUCTION

Puzzling questions when considering insects seeking food in their natural environment are: how do they cope with the complexity of the sensory information that is available, and which cues allow them to make their choices? These questions are particularly relevant for insects that have a large food spectrum, such as pollinators. This work is an attempt to answer these questions, using the honeybee–sunflower relationship as a model system.

The sunflower (*Helianthus annuus L.*) currently represents the second most important oilseed crop worldwide (Putt, 1978). The *Helianthus* genus is allogamous, with entomophilous fertilization (Free and Simpson, 1964). Honeybee visits are of great benefit for oilseed production (increased seed yield, oil rate, germination ability) (Parker, 1981). Noticeable improvement of the crop followed the discovery of male cytoplasmic sterility (Leclercq, 1969); commercially controlled hybrids were produced with heterosis benefit at the F_1 generation. Hybrid seed production is strictly dependent on cross-fertilization from male to female lines, which is brought about primarily by honeybees (Radford and Rhodes, 1978), but it is known from field observations that foragers may show a selective preference for certain genotypes, leading to low hybrid seed yields (Freund and Furgala, 1982).

In the honeybee, foraging behavior involves both learning at an individual level (Wenner et al., 1969; Masson, 1982) and communication of information to recruits (Frisch, 1967). When foraging from a flower, visual and olfactory signals (conditioned stimuli) are associated with the food reward, mainly floral nectar (unconditioned stimuli). Conditioned stimuli are memorized and become significant orientation cues. Among these stimuli, olfactory signals have been

shown to be learned particularly rapidly (Menzel, 1967; Koltermann, 1973). Thus, the foraging behavior mainly results from the association of plant allelochemicals acting as chemosensory (olfactory and gustatory) cues for the honeybee.

We have tried to determine: (1) how extensively honeybees are likely to use scents associated with a food reward to discriminate among sunflower genotypes, and (2) if all constituents of the chemical information (food and/or associated signals) are needed for host-plant recognition. Combined behavioral and chemical analyses under natural and controlled conditions were conducted.

In a first step, the distribution of forager visits on pairs of genotypes producing commercial hybrid seeds has been observed under outdoor conditions. Honeybees visit sunflowers mainly seeking nectar; little pollen is gathered (Delaude et al., 1979). Most studies dealing with the role of nectar production in the attractiveness of sunflower crops have been concerned with the relationship between the amount of nectar or total amount of sugar and bee visits (Tepedino and Parker, 1982). The quality of sugars and their relative proportions have not been taken into account. Among the four genotypes considered, no correlation has been established between the total amount of nectar or the total sugar fraction and the attractiveness of the genotypes (Fonta et al., 1985). Here we focused on the sugar composition of the nectars related to the honeybees' foraging behavior.

Little work has been done on sunflower aroma chemistry. Different types of terpenoids have been identified in the sunflower volatile fraction (Eckert et al., 1973; Gershenzon et al., 1981), but otherwise, only the aromatic value of sunflower oil has been investigated (Popescu, 1982). Our aim was first to investigate the qualitative differences between the aromas of the various sunflower genotypes and, second, to define possible cues for intergenotype discrimination by the honeybees.

Among hundreds of components detected by gas chromatography in a crude sunflower extract, 84 were indexed, among which 58 were identified, both in the polar and the apolar fraction (Etievant et al., 1984). In order to define how honeybees use such plant volatiles as orientation cues, the natural foraging situation was reproduced in a flight room, using an artificial flower feeder device (Pham-Delegue and Masson, 1985). The olfactory choices performed by the foragers were recorded subsequent to a conditioning procedure using sunflower aroma extracts. From the bees' responses, it was possible to identify those constituents among the complex volatile blend that influenced bee behavior.

From these combined studies we were able to define molecular criteria for plant attractiveness that are likely to become tools for plant improvement through entomophilous pollination.

METHODS AND MATERIALS

Chemical Cues Involved in Discrimination between Genotypes

Foraging Behavior. The experiments were conducted on two pairs of genotypes that produce commercial hybrids named Marianne and Mirasol. The seed yield reported for these hybrids differs strongly (respectively 5 – 6 quintal/ha and 10 – 13 quintal/ha). Marianne production was stopped in France and Mirasol is considered as the reference variety for sunflower breeders. Male and female lines, as well as phenological stages (stage I, II, III corresponding, respectively, to 1/3, 2/3, 3/3 of disk florets in blossom) were noted.

Observations were carried out under pollen-proof tunnels. In each tunnel a hive containing about 10,000 workers, a 1-year-old queen, brood, and food combs was placed in front of a pair of sunflower genotypes. The number of forager bees per sunflower head, as well as the stage of blossom was recorded on 40 heads per genotype, with three recordings per day, every two days during the flowering period (i.e., six days of observation on each genotype). The attractiveness of a line was reported as the mean number of foragers per 10 sunflower heads. Student t tests were used to compare the attractiveness between the parental lines of a pair.

Collection and Analysis of Nectar. Nectar samples were collected using glass pipets, from 20 florets per head and 10 heads per genotype, throughout the flowering period. The samples of nectar collected were kept in a freezer (−20°C).

The sugar composition was determined using a gas chromatography technique applied to partially dehydrated and derivatized samples (Fonta et al., 1985). To measure the real proportion of the constitutive sugar, an internal standard (triphenylethylene) was added to the samples (Black and Bagley, 1978).

Collection and Analysis of Aromas. Plant volatiles were collected and concentrated using a head space trapping method adapted to sunflower heads (for details, see Pham-Delegue et al., 1989). Aroma collection was carried out (1) on the four genotypes at three phenological stages (I, II, III), to evaluate the stage effect on the production of volatiles and (2) on the four genotypes at stage II, to focus on the sex effect (female versus male lines).

Statistical analysis of the stage effect was performed on the integrated area of each constitutive peak as a ratio of the total area of all indexed peaks of the chromatogram of a given genotype. Data were then compared for each pair of sunflower lines (two repetitions per stage and two injections per sample), using an univariable analysis of variance followed by a multiple comparison of means (Scheffe method).

The data treatment of the sex effect was applied to the values of the integrated area of each indexed peak as a ratio of standard peak area (1 mg

2-hexanone added before concentration of extracts). To compare male to female aromagrams (three repetitions per genotype and two injections per sample), a univariable analysis of variance was used.

For identification of the discriminative components, gas chromatography and mass spectrometry were coupled, following conditions previously described (Etievant and Bayonove, 1983; Pham-Delegue et al., 1989).

Processing of a Complex Chemical Signal

Bioassay. A honeybee colony (about 3000 workers, and 1-year-old queen and brood) was kept in a flight room under controlled conditions (Temperature 25°C; relative humidity 55%; 12-hr light–12-hr dark). Foragers were allowed to visit a device (during a 2-hr period) on which a sugar solution was placed (sucrose 50%, the unconditioned stimulus), and a scent (the conditioned stimulus, successively consisting of the total sunflower extract and its polar fraction, see below).

Bees then were tested, without food reward, in a choice situation between the conditioned scent and an unconditioned scent stimulus. The test period was divided into four trials of 5 min, interspersed with periods in which the conditioned scent was again presented with a food reward. The distribution of visits during the test periods expressed as a percentage of landings for the different stimuli examined, was compared using a χ^2 test, 1 *df.*

Chemical Stimuli. To provide general information on sunflower aroma constituents, a bulk sample was made of 10 flower heads from various cultivars. The sunflower heads were collected and extracted using a dichloromethane solvent extraction method, which has been proven to be as efficient as the headspace method (Etievant et al., 1984). The solvent extract was first separated into polar and apolar fractions, and then the polar fraction was arbitrarily split into three fractions (A, B, C) as described in Pham-Delegue et al,. (1986). Separation and identification methods have been described previously (Etievant et al., 1984).

RESULTS

Chemical Cues Involved in Discrimination between Genotypes (Figure 1)

Foraging Behavior. A foraging preference appeared for the flowering stages I and II, stage III being visited much less often in all genotypes. Moreover, the foragers' visits were randomly distributed between the parental line of Mirasol, whereas the female line of Marianne was visited significantly more often than the male line.

Thus, the honeybees' ability to discriminate between genotypes and flow-

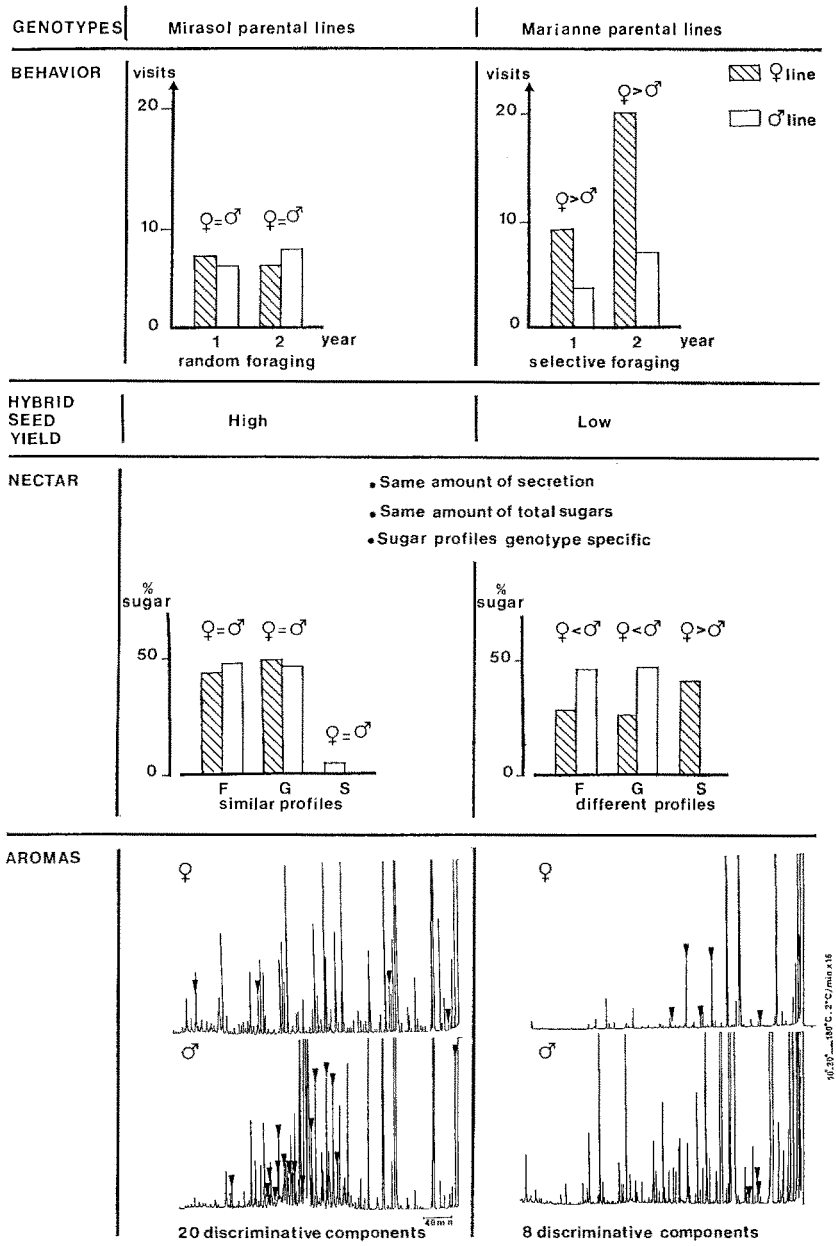


FIG. 1. Synopsis of chemical cues (nectar, aromas) involved in discrimination between genotypes (F: fructose; G: glucose; S: sucrose).

ering stages (Drane et al., 1982) was confirmed. It has to be emphasized that the selective behavior performed on Marianne parental lines resulted in a low seed yield, whereas the random distribution on Mirasol parental lines contributed to a high level of seed production.

Analysis of Nectar. Fructose, glucose, and sucrose were identified. All genotypes present high levels of glucose and fructose that are equivalent (30–50% of the total sugars), but the proportion of sucrose fluctuates strongly between genotypes: 40% sucrose was measured in the female line of Marianne, which was visited far more than the male line, where no sucrose was detected in the nectar. In Mirasol parental lines, only traces of sucrose could be found in both lines, corresponding to an equal distribution of visits. We may thus assume that the sugar composition of nectar, particularly the proportion of sucrose, is a key parameter in the honeybees' selective foraging behavior. However, although bees' preferences correlate well with the quality of the food reward available, bees are able to recognize the preferred genotype using distant chemical cues, i.e., aromas.

Analysis of Aromas. Of the 144 components indexed for Marianne lines and 136 components for Mirasol lines, 17 of the components for Marianne lines and 18 for Mirasol lines differed significantly according to flowering stage. Nine of the 17 variable components in the Marianne lines showed higher relative proportions at stage I, and 13 of the 18 variable components of Mirasol lines showed higher relative proportions at stage II. These data can be related to the behavioral observations in which bees showed preferences for flower at stages I and II for all genotypes.

To compare aromas from male and female lines, 134 and 250 peaks were indexed for Marianne and Mirasol lines, respectively. Significant differences appeared in eight of the 134 components for Marianne lines and in 20 of the 250 components for Mirasol lines. It has to be stressed that differences between genotypes do not necessarily occur in components that are most abundant. Three of the eight and 12 of the 20 variable components for Marianne and Mirasol lines, respectively, appear only in one or the other genotype of a pair, generally in the male line. Moreover, most of the variable components between Mirasol lines have higher amounts in the male line (16 of 20 components). The identification of some of the discriminative components has been reported elsewhere (Pham-Delegue et al., 1989).

Processing of a Complex Chemical Signal (Figure 2)

After being conditioned to the total extract, the bees were placed in a choice situation between the conditioned stimulus and either the polar or the apolar fraction of the total extract. Behavioral responses showed that the apolar frac-

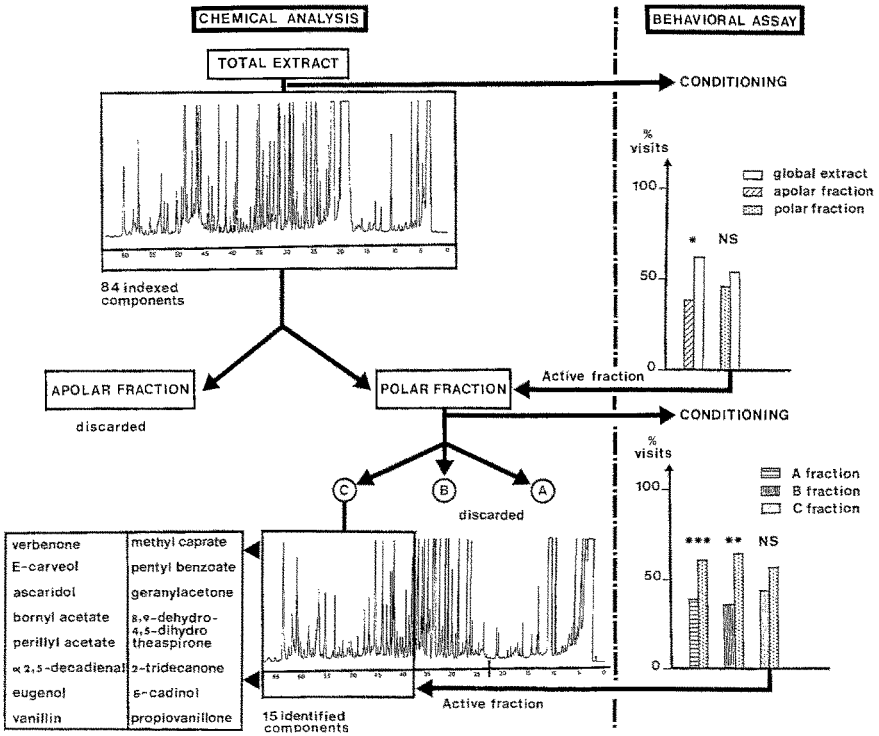


FIG. 2. Scheme of the combined chemical and behavioral procedures leading to the identification of active components that allowed the recognition of the sunflower volatile blend by the forager bees (χ^2 test, 1 *df*. NS: not significant; *P < 0.05; **P < 0.01; ***P < 0.001).

tion was significantly discriminated from the conditioning scent, while the polar fraction was confused with the global aroma. Thus the polar fraction was considered by the bees as very similar to the global extract.

Following the same procedure, the three fractions of the polar extract were presented to the foragers previously conditioned to the polar extract. It appeared that only the C fraction was statistically confused with the conditioned aroma, whereas the A and B fractions were significantly less attractive ($P < 0.001$ and $P < 0.01$, respectively) than the whole polar fraction. The C fraction includes 28 constituents, among which 15 were chemically identified, the others being sesquiterpenes (Pham-Delegue et al., 1986). This C fraction, the so called active fraction, represents the most restricted fraction of the sunflower volatile blend that elicits the same level of attraction as the total extract in our experimental conditions.

DISCUSSION

From the study of the honeybee–sunflower model, some information can be gained on the role and the nature of plant chemicals involved in host plant selection and on subsequent applications.

Nectars appear to be the key factor influencing honeybee choices. As is the case in all floral nectars, sunflower nectars consist mainly of sugars with simple qualitative profiles. Glucose, fructose, and sucrose were identified, in proportions characteristic of the genotype (Pham-Delegue et al., 1987); this was later confirmed within a larger range of sunflower genotypes (Vear et al., 1990). Moreover, the level of visits appeared to be positively correlated with the amount of one particular sugar sucrose (Pham-Delegue et al., in preparation). Thus we may assume that a plant breeding program to increase the amount of sucrose would lead to an increased attractiveness of the genotypes towards forager bees. An increased level of pollinator visits would increase the speed of seed maturation and the oil rate (Parker, 1981). Furthermore, the sugar composition of nectars in the genotypes to be paired for hybrid seed production could be screened out easily using gas chromatography (Fonta et al., 1985) or liquid chromatography methods (Erickson et al., 1979; Severson and Erickson, 1983; Vear et al., 1990) in order to avoid the pairing of genotypes with different amounts of sucrose.

Currently plant breeders, who face many other agronomic constraints, do not consider the sugar composition of nectars as an attractant to pollinators, either for screening procedures of genotypes or for more basic genetic studies. However, the effects of plant breeding on nectar secretion to improve entomophilous cross-pollination are taken into account in some species such as oilseed rape *Brassica napus* L. In this species, genotypes with initial depressed nectar production had their secretion restored through selection to increase pollinator attraction (Mesquida et al., 1988). We consider work directed towards increased nectar amounts or sugar amounts in nectar secretions as the most promising way to improve cross-pollination using insects. Other parameters, such as composition of amino acids, proteins (Baker and Baker, 1976), and ions (Waller et al., 1972), also may have an important influence on bees' choices.

Long-distance orientation cues such as aromas are associated with the food reward. In the sunflower, as in many other species [e.g., cotton (Hedin, 1976) or maize (Buttery et al., 1978)], the aromas are very complex mixtures whose composition is plant-specific; most constituents are common to all sunflower genotypes. From a restricted, active fraction of the common constituents, bees are able to generalize the total plant aroma. However, slight differences between the aromas of the different genotypes, as shown by comparing their chromatographic profiles, may be used as discriminative cues that enable foragers to select the best food source. The identification of active fractions and of com-

ponents specific to given genotypes would be of interest for plant breeders. It may be possible to combine plant genetic and chemical analyses to elucidate the genetic basis of volatile biosynthesis. This may aid the development of plants that produce an attractive aromatic blend, or, alternatively, to develop plants that are chemically resistant towards pest insects. Many studies have been conducted to identify plant chemicals acting as attractants or repellents towards pest insects (e.g., Finch, 1977; Visser et al., 1979; Gershenzon et al., 1981; Guérin et al., 1983; Dawson et al., 1989), but very limited work has been devoted to the chemicals involved in pollinator attraction. By combining behavioral and chemical analyses, we have shown that honeybees could use a reduced fraction of the total aroma to recognize the sunflower blend. Further studies using a direct coupling between gas chromatography and electroantennogram recordings have indicated that a limited range of components elicit the highest antennal responses (Thiery et al., 1990). As a matter of fact, investigations to identify volatile components involved in bee attraction may result in the identification of a range of active components but probably not as limited as plant chemicals involved in host recognition in pest insects. This may depend on the different behavioral repertoires of both kinds of insects. Taking into account the learning abilities of the honeybees, we may assume that they will not limit their response to very specific plant volatiles since they can learn to respond to other less specific components when they are associated with a food reward.

Elsewhere, attempts were made to use these learning abilities to improve the visits of forager bees to a target plant using either conditioning of bees to floral scents (Gühler, 1930; Koch, 1931; Burgett, 1980) or attraction mediated by pheromonal blends (Williams et al., 1981a,b). Such methods are of interest, particularly when a great amount of honeybee visits is needed on a crop during a short period of time. However, if long and regular foraging behavior is needed to obtain cross-pollination, then the use of olfactory stimuli will not be efficient if the food provided by the crop does not reward the bees. Another way of using the behavioral plasticity of honeybees would be to modify their olfactory preferences at early stages of their development. It has been shown that the honeybee antennal system becomes functional three days before emergence and that olfactory rearing conditions during preimaginal stages to early adult day-life could affect antennal sensitivity (Masson and Arnold, 1984) and synaptic density in the antennal lobe (Gascuel and Masson, 1987). Olfactory exposure at early adult stages was shown to induce behavioral changes towards the exposed odorant (Pham-Delegue et al., 1990). These data suggest that changes in the olfactory environment applied during a sensitive period may lead to predictable and durable behavioral changes. The possible application of this for controlled pollination using floral components as exposure stimuli must be considered.

Finally, a better knowledge about the allelochemicals involved in plant–

pollinator relationships will result in the determination of molecular criteria influencing honeybee selective foraging behavior. Such chemicals could be used either sprayed on the crop to attract pollinators with or without previous conditioning or produced by the target plants after integration into plant breeding programs. This latter field of experiments, which requires cooperation between geneticists, plant breeders, insect physiologists, and biochemists, seems to be the most promising prospect for new applications in the control of entomophilous pollination.

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SEMIOCHEMICALS FOR USE WITH PARASITOIDS: STATUS AND FUTURE

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Abstract—Allelochemicals are known to serve important roles at all steps in the host-searching sequence of parasitoids. We discuss the various roles of these allelochemics and the type of information needed to develop their use in pest control, which to date has been very limited. Rapid advancements are being made with respect to airborne chemicals and longer-range foraging behavior. Moreover, recent discoveries have shown that genetic diversity in parasitoid populations and phenotypic plasticity of individuals, together with their physiological state, often result in substantial variations in the response to chemical cues. Successful application of semiochemical-parasitoid systems will require management of these intrinsic parasitoid variables as well as management of the foraging environment. We illustrate emerging technology for such an application. For the immediate future, the development of this technology will allow us to: (1) define the genetic and phenotypic foraging profiles important to consistent and efficient parasitoid foraging, and (2) establish the proper propagation and release procedures and monitoring bioassays necessary to ensure appropriate behavioral and physiological qualities of released organisms. For the long term, we envision technology for comprehensively manipulating the pest/crop environment in ways that would provide foraging stimuli and other needs important to retention and efficiency of parasitoids.

Key Words—Semiochemicals, kairomones, parasitoid behavior, parasitoid manipulation, foraging environment, *Heliothis zea*, Lepidoptera, Noctuidae, *Microplitis croceipes*, Hymenoptera, Braconidae.

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INTRODUCTION

Semiochemicals are known to serve a major role as cues to aid parasitoids in locating and recognizing their hosts. Thus, a knowledge of these chemicals and their functional roles and mechanisms is an important consideration in the effective design of all types of programs for the use of parasitoids as biological control agents. In this report we discuss the sources of semiochemicals affecting the host foraging behavior of parasitoids, their primary functional roles, and their status and future prospects for the enhancement of parasitoids as pest control agents. A number of earlier reports have addressed these subjects to various extents. However, substantial recent progress, particularly on the role of airborne chemical cues and plasticity of parasitoid responses, makes an update worthwhile. We will not attempt to cover the subject of sex pheromones of parasitoids or semiochemicals used by adult parasitoids in food location. We also will not attempt to review all the literature on the subject of host-foraging semiochemicals. Rather, our intent is to discuss briefly the components necessary for designing effective biological control programs and then use recent representative data from studies of the *Heliothis* spp. larval parasitoid *Microplitis croceipes* (Cresson) to illustrate the emerging new prospects for semiochemicals as tools to enhance parasitoids as biological control agents.

GENERAL TRITROPHIC CONSIDERATIONS

An abundance of chemical communication is involved in mediating interrelations among habitat, host, and parasitoid trophic levels (Price et al., 1981). Many interspecific and intraspecific interactions at all trophic levels are mediated by chemical cues. Thus, both allelochemicals (interspecific) and pheromones (intraspecific) are involved (see Nordlund and Lewis, 1976; Dicke and Sabelis, 1988 for discussions of terminology). The various chemical mediators may have multiple functions in the network of interactions, and these functions are not necessarily mutually exclusive. For example, a marking pheromone or sex pheromone of the host may be used by the parasitoid as a host-finding kairomone, and a given plant odor may be used by both a plant-feeding host and its parasitoid as habitat attractants. We will be exploring the cues used by parasitoids and their uses in pest control. However, other roles of semiochemicals and the net effects of their use must be considered as well.

ORIGIN AND FUNCTION OF SEMIOCHEMICALS MEDIATING HOST LOCATION

Once a female parasitoid has entered a suitable habitat, she must locate a potential host. This part of the host-selection process is termed host location (Vinson, 1976) and can be defined as the perception and orientation by para-

sitoids to their hosts, from a distance, by responses to stimuli produced or induced by the host or its products (Weseloh, 1981). The role of semiochemicals in this process has been well documented (e.g., reviews by Vinson, 1976, 1984; Weseloh, 1981; Lewis and Nordlund, 1985), and these chemical cues appear to be critical to the successful location of hosts by many parasitoid species.

In a given host–plant–parasitoid complex, semiochemicals important in the host-location process may be produced at more than one location. The plant upon which the host is feeding, the host itself, or products of the host all release chemicals that can serve as mediators of host location. In addition, many species of parasitoids use chemical markers to distinguish between parasitized and unparasitized hosts. These chemicals are important cues to enabling parasitoids to avoid superparasitism, although superparasitism may be adaptive in some circumstances (Dijken and Waage, 1987). Knowledge of the source of these semiochemicals and how they govern the behavior of foraging parasitoids is necessary if we are to utilize these chemicals to enhance the effectiveness of biological control agents. It should be emphasized here that chemical (and nonchemical) host location cues for a given species of parasitoid are not mutually exclusive; an individual may be required to use any or all of the available resources to find a potential host. In this section we examine habitat, host insect, and marking cues as sources of semiochemicals that mediate host location by insect parasitoids.

The Habitat

Besides playing an important role in host habitat location (Vinson, 1981), chemical cues released directly by the plant can be important in host location. For example, chemicals released by artificially damaged plants have been shown to elicit positive responses from *M. croceipes* (Eller et al., 1988), *Campoletis sonorensis* (Cameron) (Elzen et al., 1983), and *Cotesia* (=*Apanteles*) *marginiventris* (Cresson) (Loke et al., 1983).

More commonly, however, chemicals that are released as a direct result of host activities or chemicals that have been sequestered or otherwise modified by the host have been shown to be important host-finding cues. In many cases, parasitoids have been shown to prefer plant material that has been damaged by a host insect to artificially damaged material (Vinson, 1975; Loke et al., 1983; Drost et al., 1986, 1988; Turlings et al., 1990). These studies suggest that volatile chemical cues are released as a direct result of host feeding activity, involving a blend of plant- and host-derived compounds. In one case, quantifiable differences in chemical composition of volatiles produced from leaves damaged by two different species of noctuid larvae have been shown (T.C.J. Turlings, personal communication). Further, *C. marginiventris*, a parasitoid that attacks both of these species, has the ability to distinguish between damage caused by each of the larval hosts.

Plant-derived chemicals also may be incorporated into the body of the host or host-related products and further influence parasitoid behavior. For example, *Microplitis demolitor* (Wilkinson) reared from cowpea-fed *Heliothis zea* (Boddie) larvae will respond to infested cowpea plants, while parasitoids reared from artificial diet-fed larvae will not (Hérard et al., 1988). Evidence suggests that a plant-derived chemical(s) is present in the cocoon of the developing parasitoid and that contact with this chemical(s) at time of eclosion increases the responsiveness of the adult parasitoid to certain plant odors (Hérard et al., 1988). The phenomenon in which preadult experience influences the adult organism has been termed the "chemical legacy hypothesis" (Corbet, 1985).

Perhaps the best known examples of plants as a source of kairomones have involved studies of the parasitoid response to host larval feces. For example, females of both *M. croceipes* (Sauls et al., 1979) and *M. demolitor* (Nordlund and Lewis, 1985) show a much stronger response to feces from cowpea-fed *H. zea* larvae than to feces from diet-fed larvae. In the case of *M. croceipes*, a key active component in *H. zea* larval feces, and apparently not diet related, has been identified as 13-methylhentriacontane (Jones et al., 1971). Plant-derived compounds in host larval feces may be simply excreted unchanged or they may be chemically altered as they pass through the digestive system. Regardless, it is apparent that, in many cases, host diet can influence the behavioral response by a parasitoid. This effect can include diet from different plant species, different varieties, cultivars, plant ages, or even different tissues from the same plant (e.g., flowers, leaves, etc.).

The Host Insect

Besides feces, several host-related products have been shown to contain chemical cues that mediate host location by parasitoids. Scales from adult *H. zea* that are rubbed off during oviposition can serve as a host-finding cue for the egg parasitoid *Trichogramma evanescens* Westwood (Lewis et al., 1971, 1972; Gueldner et al., 1984). Oral secretions from feeding or silking larvae have been shown to contain host-seeking cues for several parasitoids. (Vinson and Lewis, 1965; Weseloh, 1977; Ding et al, 1989). Other host products, such as marking pheromones (Prokopy and Webster, 1978), aggregation pheromones of bark beetles (in combination with volatile tree host terpenes) (Camors and Payne, 1971, 1973; Borden, 1974, 1977), and sex pheromones (Lewis et al., 1982; Nordlund et al., 1983; Noldus, 1988; Noldus et al., 1989), can contain active volatile chemicals used by parasitoids as host location cues.

Host Marking Cues

Finally, we want to consider briefly host marking cues and their importance to biological control programs. Host discrimination, or the ability of a

parasitoid to distinguish between parasitized and unparasitized hosts, is a well-documented phenomenon (see Lenteren, 1981, for review). In some cases, the parasitoid leaves a chemical marker on or near the host during oviposition; in other cases the condition of the host is altered as a result of parasitization. Generally, these cues are thought to function as a means for the parasitoid to avoid revisiting sites and reparasitizing a host, thereby reducing foraging efficiency. A knowledge of the source and function of these chemical and/or physical cues is important to elucidating their foraging behavior and thereby assist in managing parasitoids as biological control agents.

DYNAMICS OF THE SYSTEM

Environmental Variations

The habitat and host resources with which a parasitoid species or population must deal are far from static. Rather, the combination of these two trophic levels may vary greatly both within and among parasitoid generations. For example, in the case of a parasitoid that attacks a plant-feeding host, the plant's environment may vary in any combination of species, age, growth pattern, part upon which the host is feeding, etc. Superimposed on these plant variations may be hosts of different species, ages, densities, distributions, etc. Furthermore, the microclimatic and macroclimatic conditions are subject to variations within and among habitats. The suitability of the environment and the quantity and quality of semiochemical information for the parasitoid will, in turn, vary with these circumstances.

Lewis and Gross (1989) showed that the *Heliothis* larval parasitoids *M. croceipes* and *Cardiochiles nigriceps* (Viereck) were substantially more efficient at locating their hosts on some plants than on others. Lewis et al., (1972) reported considerable differences in the rates of parasitism by *C. nigriceps* of *Heliothis virescens* (L.) larvae of different ages and of different parts of the plant within a single field of cotton. Numerous researchers have incorporated such considerations into predictive models of various types to assess impact on the parasitoid's foraging efficacy and pest populations (see Hoy and Herzog, 1985).

A number of reports have specifically explored the effects of these plant and host variations on chemically mediated parasitoid behavior. Drost et al. (1988) demonstrated in flight chamber tests that females of *M. croceipes* have varying preferences for odors of different plant species, ages, and parts. The vast array of cultivars resulting from modern plant breeding programs and practices add to such variations. Elzen et al. (1986) and Martin et al. (1990) demonstrated significant differences in the semiochemically mediated behavior of a hymenopterous and dipterous parasitoid, respectively, to different cultivars of cotton.

Parasitoid Variations

As a consequence of environmental variations and other causes, substantial diversity is a common feature among individuals of a parasitoid species or population. Intraspecific diversity in semiochemically mediated behavior is no exception. Three categories of intrinsic variations in parasitoids will be considered here: (1) genetic diversity, (2) phenotypic plasticity, and (3) physiological state.

Genetic Diversity. Genetic variations in the traits of a parasitoid species is an established phenomenon known to be important to biological control (Mackauer, 1976). Populations of a parasitoid species with long-standing associations with different hosts and/or habitats are known to differ in their affinity and behavior relative to those host-habitat situations (Mollema, 1988; Pak, 1988). Prevost and Lewis (1990) demonstrated measurable heritable differences in the semiochemically mediated responses of females of a single laboratory colony of *M. croceipes* that had been in culture for over two years. Reliable use of parasitoids requires a knowledge of the relative value of these different traits and an ability to manage their respective occurrence. Other than a few general discussions on the subject, including those of Boller (1972) and Huettel (1976), very limited attention has been given to assessing and monitoring quality control of behavior in parasitoids cultured for use in biological control.

Phenotypic Plasticity. Recent studies have shown that the behavior of individual parasitoids, including their response to semiochemicals, can be modified substantially through learning (Alphen and Vet, 1986). Studies show that many species of parasitoids are able to acquire, by experience, an increased preference for, and/or ability to forage in, a particular environmental situation. The learning process is often associative learning where the parasitoid learns to use a previously weak or neutral cue for host foraging by associating it with the host or host product (Lewis and Tumlinson, 1988; Vet et al., 1990; Turlings et al., 1989). This plasticity is an important adaptation that enables the parasitoid to make adjustments for fluctuations in foraging situations within and among generations. Other authors cover this subject in detail in this issue. However, for the sake of clarity of our applied discussion, it is important to cover several essential items in more detail.

An important learning process for *M. croceipes* was found to occur when females antennate the feces of their larval host. During antennation they learn and are subsequently attracted to volatile plant odors associated with the host by linking the volatiles to a host-specific recognition kairomone in the feces (Lewis and Tumlinson, 1988). Such learning can begin at or just before eclosion based on the host products associated with the parasitoid pupa (Vet, 1983; Hérard et al., 1988). Thus, the methods of rearing the parasitoid, particularly host species and host diet, can have a major impact on their responsiveness to semio-

chemicals important in their host selection behavior. For instance, rearing *M. croceipes* from hosts fed on artificial diet may result in a significant reduction in the parasitoid's ability to respond to natural host plant odors, compared to parasitoids reared on hosts fed a diet of plant material (Hérard et al., 1988). After reaching adulthood, the parasitoid's foraging responses are modified continually according to the foraging circumstances encountered (Vet et al., 1990). Wackers and Lewis (1990) found that *M. croceipes* learns to differentiate between odors of different plant parts and prefers the most profitable odors based on oviposition encounters. Therefore, the prospects and need for using semiochemicals to manipulate the foraging behavior of parasitoids in the field becomes an important consideration.

Physiological State. A parasitoid's physiological state relative to needs such as food, mating, and general health can strongly influence its propensity and ability to respond to host selection cues. For example, if a female parasitoid is hungry, the appetitive drive for food cues may take precedence, and, as a result, responses to host-related cues may be reduced (Hagen and Bishop, 1979). Also, a lack of mature eggs in the ovaries can reduce its response to olfactory cues (Shahjahan, 1974). Further, the presence of other strong chemical, visual, or auditory cues would likely disrupt its response to host-foraging cues by dilution. The physiological state of parasitiods relative to other needs can be viewed as a gateway that filters the detection and prioritizes responses to foraging stimuli based on current needs. The influence of a parasitoid's physiological state on foraging behavior has generally been recognized as important, but has received little systematic study.

APPLIED CONSIDERATIONS

Two features of parasitoid behavior are essential for effective foraging; (1) efficient location and attack of their hosts, and (2) retention of females in the target area. To meet these requirements we must understand and manage the factors that influence the foraging behavior of the parasitoid. Dependably effective performance by parasitoids is a product of properly matching the intrinsic conditions of the searching female with the target environment (Figure 1). The ability to manage the environmental component of the interaction is important in all approaches for using parasitoids, including enhancement of wild populations as well as maximizing the performance of laboratory reared and released parasitoids. On the other hand, management of intrinsic variations in the parasitoid response behavior is more applicable in situations where parasitoids are laboratory reared and released.

The steps involved in a propagation and release program and the problems and needed technology for managing these problems are shown in Figure 2.

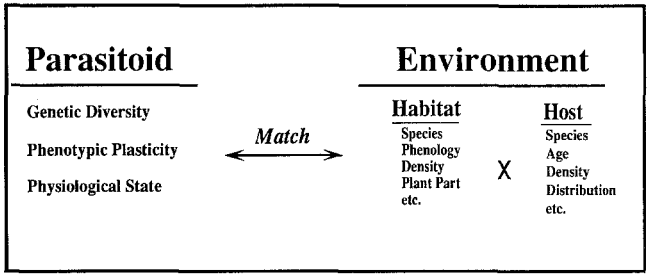


FIG. 1. Sources of variation in the parasitoid and its foraging environment which need to complement each other in order for effective foraging to occur.

Managing the Parasitoid Component

We have discussed various aspects of genotypic and phenotypic diversity among and within parasitoid individuals that contribute to substantial variability in their foraging behavior. In the case of natural parasitoid populations, natural selection is operating continuously to select and shape the features most effective for that environment. However, by laboratory colonization we remove the parasitoid from the context of natural selection and place it into an artificial environment that may change genotypic frequencies and phenotypic conse-

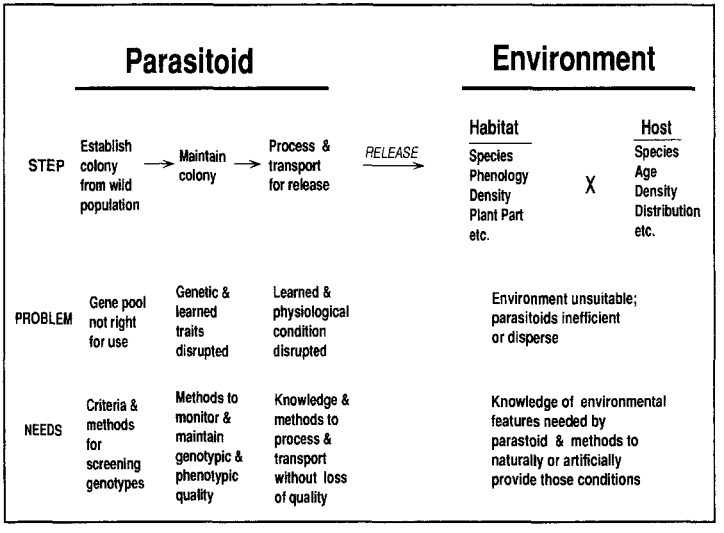


FIG. 2. The problems and associated technology needed in the indicated steps of propagation and release of parasitoids for effective pest control (the genotypic and phenotypic behavioral profile of the parasitoid must match with certain environmental conditions.

quences (Wardle and Borden, 1986; Hérard et al., 1988). These consequences are of particular danger in the case of inundative and seasonal inoculative programs where propagation and release are continuously artificial and the genotypic and phenotypic traits of the field population are dependent upon their prior laboratory colonization conditions (Lewis et al., 1990). In the case of inoculative releases, there is still an important need to manage the quality of propagated and released parasitoids, although perhaps it is less critical than in inundative releases. In these inoculative cases, natural selection "screens" the traits of the released parasitoids for the effective components for establishment. However, proper management of the colonized and released insects could greatly increase the success and speed of establishment.

The knowledge of features critical for parasitoid foraging behavior and mechanisms governing those features is a newly emerging area. Thus, we cannot yet provide a specific prescription for monitoring and managing the variables during the production and release of parasitoids. Rather, our objective is to provide a conceptual framework for developing the necessary knowledge and to illustrate the plausibility of the technology.

We must expand our appreciation of the need for quality control procedures in the establishment, maintenance, and use of colonized parasitoids and develop methods for implementing these procedures. The quality control considerations will have to include both genotypic and phenotypic aspects of subtle but important behavioral traits and the significant but not readily apparent ways that various rearing and release methods might affect these traits.

Genetic Qualities. When selecting wild material of a parasitoid species for establishing a founder laboratory colony, we need to screen the diversity of genotypic traits and ensure that the traits of the colonized population are appropriately matched with targeted use situations. To do this, we must further develop bioassays that can be used to evaluate the diversity, behavior, and other traits of the parasitoid. Successful parasitism of a target host in a confined situation does not guarantee that released individuals will parasitize that host under field conditions. The sequence of host selection behaviors may be circumvented in laboratory confinement. Various techniques and equipment, such as olfactometers (e.g., Vet, 1983; Vet et al., 1983), flight tunnels (e.g., Drost et al., 1986; Zanen et al., 1989), ranked behavioral assays (e.g., Lewis and Jones, 1971; Wilson et al., 1974), and small field assays (e.g., Keller and Lewis, 1989), can be used in screening and selecting material for establishing the colony. The tests should be representative of the climatic, habitat (e.g., host plant species, age, and plant part), and host insect (e.g., age, density, and distribution) situations that the parasitoid will encounter under field situations, and the tests should be adequate for evaluating the parasitoid's ability to perform the full sequence of host selection behaviors in those situations. A good balance is needed between enough genetic diversity (to cope with the fluctuations they

will encounter) and not too much diversity (for consistency). However, the amount of diversity desired may vary among the different traits. Similar testing techniques to monitor the colony systematically are needed for preservation of the diversity and uniformity of these traits. The report of Prevost and Lewis (1990) provides an example of how a flight chamber can be used to assess various genetic variations in host-finding response traits and how they can be measured and compared over generations for different colony lines.

Phenotypic Qualities. The response potential of a parasitoid is often a result of learning experiences in both the preadult and adult stages. Without care, insectary environments can create either weak or distorted response profiles. However, by understanding the sources and mechanisms of learning, we can provide the appropriate level of experience. As discussed earlier, the lack of important semiochemicals in the host insect diet and/or use of factitious host insects have been shown to cause poorly responsive parasitoids. These semiochemicals can be incorporated artificially into the diet and/or on the hosts as synthetics or as materials such as plant extracts. This approach may be particularly important in cases where important learning experiences occur in the immature or early adult stages (Hérard et al., 1988; Wardle and Borden, 1985). Another approach that has been the subject of some experimentation is the prerelease exposure of the adult to important stimuli. Gross et al. (1975) showed that with *M. croceipes* and *Trichogramma pretiosum* Riley, exposure to host feces or host moth scales, respectively, increased the proportion of parasitism in the release area and attributed the benefit primarily to a reduction in the escape response upon emergence from the release container. Subsequent studies, discussed earlier in this paper, have shown that prerelease exposure of the parasitoid to the kind of host and associated stimuli situations that they will encounter in the field can result in associative learning that enhances the parasitoid's ability to perform after release.

There are genetic variations among individuals of a species that influence what can be learned and to what degree. These variations are an important consideration for both the genotypic and phenotypic qualities of parasitoids.

Physical and Physiological Qualities. In addition to the constraints placed on learning experiences, the unnatural insectary environment can be stressful to the physical and physiological well-being of parasitoids. For example, insect movements are restricted, and unnatural lighting, temperature, and humidity may affect the females in a way that subsequently alters foraging behavior. P.O. Zanen (personal communication) found that chilling the cocoons of *M. croceipes* severely reduced the ability of females to make flight responses to volatile host odors. Sublethal diseases not readily apparent may spread in the colony and affect behavior. Hamm et al. (1988) reported a viral infection in an *M. croceipes* colony that accounted for reduced host finding responses in flight

chamber studies. Further, mating and nutritional conditions can affect foraging responses strongly.

As is apparent from these points, the physical and physiological needs are very important to effective foraging behavior. Since neither weaknesses in the learning conditions nor those of the physical or physiological state may readily be apparent from general observation, various response evaluation techniques such as those discussed for the genotypic traits should be employed to monitor the quality of phenotypic traits of the colony.

Managing the Environmental Component

Given the release of parasitoids of adequate quality into the habitat and/or the presence of wild parasitoids of proper quality, we can then manage the environment so as to maximize performance. As stated earlier, two basic objectives must be achieved: retention of the parasitoids in the target area and efficient host search and attack behavior. For this to occur, the host plant or other habitat substrate must be suitable and there must be a sufficient host density. Otherwise the parasitoids will not remain in the area. The economic damage threshold of the pest can be at a density below that needed to sustain effective parasitoid foraging behavior. Semiochemicals offer good prospects as a tool for managing the parasitoid behavior independent of these variables.

Hagen et al. (1970) first manipulated a bollworm natural enemy with a semiochemical by using an artificial honeydew to attract adults of *Chrysoperla carnea* (Stephens). These chemicals provided a kairomone and food supplement, both of which served to increase predator density. Indole acetaldehyde, a breakdown product of tryptophan present in the yeast hydrolysate of the artificial honeydew, operated as a kairomone by attracting adult lacewings into target fields (Emden and Hagen, 1976). Other components of the artificial honeydew (sugar, water, whey–yeast hydrolysate) arrested movement of the lacewings and served as a nutritional supplement, thereby promoting oviposition. One week following an application of such a food spray to cotton, *Chrysoperla* egg density increased from one to three per plant, and both the density of bollworm eggs and the number of damaged bolls declined (Hagen et al., 1970).

The use of semiochemicals from both plants and the host, *H. zea*, has been shown to increase rates of egg parasitism by *Trichogramma* in the field. For example, parasitism of eggs of *H. zea* by *Trichogramma* spp. increased from 13% in control plots to 22% in soybean treated with an extract of scales collected from *H. zea* (Lewis et al., 1975a, b). Similarly, the release of a synthetic blend of the sex pheromone of *H. zea* in cotton increased parasitism of eggs from 21% in control to 36% in treated plots (Lewis et al., 1982). Altieri et al.

(1981) demonstrated that spraying various plant extracts onto crops can stimulate increased rates of parasitization. For example, parasitism of eggs of *H. zea* by *Trichogramma* spp. was 21% on soybean treated with an extract of *Amaranthus* compared to 13% on plants sprayed with water. The behaviors that lead to increased parasitism by *Trichogramma* in the presence of either plant extracts or sex pheromones are not yet fully understood.

Proper application of plant-derived synomones may attract parasitoids and reinforce their activities in habitats where these cues are absent, weak, or improperly distributed. By using aqueous extracts of *Amaranthus* or corn, Altieri et al. (1981) found they could significantly increase rates of *Trichogramma* parasitization of host eggs in soybean, cowpea, tomato, and cotton. Once attractive plant chemicals and underlying response mechanisms are elucidated, synthetic behavioral chemical applications, plants breeding schemes, or genetic engineering could be used to enhance the attractiveness of certain cultivars to parasitoids.

Application of semiochemicals to crops has not been universally successful in stimulating increased rates of mortality. For example, the use of a uniform spray of moth scale extracts may reduce egg parasitism by *Trichogramma* spp. at low host densities, apparently by stimulating females to search too intensively where no hosts are present, thereby lowering their efficiency (Lewis et al., 1979). This problem can be partly overcome by impregnating particles of diatomaceous earth with the moth scale extract to mimic natural scales (Lewis et al., 1979). When dispersed through a field, the treated particles stimulate intermittent, rather than continuous, searching by *Trichogramma*.

From these and other studies, it is obviously important to understand the respective roles of the various cues in the host selection sequence and to apply the long- and close-range cues in proper proportions and distribution to effectively retain the parasitoid in the desired habitat without interfering with efficiency. The vacuum in understanding long-rang foraging behavior is an obstacle in achieving these needs. However, recent research is breaking through these barriers. In the next section we present specific data from small-plot field studies to illustrate how emerging knowledge can be assembled into an applied biological control package.

Other Applications

We have dealt primarily with host location mediators and their use. However, another area of cues and potential application that we should mention is host recognition and oviposition cues. Defining the chemical as well as the physical factors mediating host recognition and acceptance will be an essential component for allowing us to induce parasitoids to oviposit in surrogate host

media for in vitro rearing. For example, Nettles et al. (1982) showed that solutions of $MgSO_4$ and KCl, when encapsulated in small spheres simulating host eggs, will elicit oviposition by *T. prestiosum*. Tilden and Ferkovich (1988) reported that *M. croceipes* could be induced to oviposit in agarose drops soaked with hemolymph from *Heliothis* larvae. They and Heath et al. (1990) reported progress on the characterization of this oviposition-stimulating kairomone.

As was pointed out in the discussion of tritrophic considerations, semiochemicals are a common thread mediating behavioral interrelationships among plants (or other substrates), phytophages (or other primary consumers), and parasitoids. Most research efforts have been directed separately toward their utilization as tools to disrupt the reproductive or feeding activities of the pests, or to enhance the performance of parasitoids. However, we encourage a greater consideration of the prospects for multitactic employment of semiochemical packages. These multitactic packages could include various combinations such as mating, oviposition, and feeding deterrents for the pests together with formulations to increase attack by parasitoids. Such packages will require a thorough understanding of the individual and multiple functions of the chemicals since their roles often overlap. The reports by Lewis et al. (1982, 1985) and Noldus and van Lenteren (1985a,b) demonstrating the use of host sex pheromones and marking pheromones as host-finding kairomones by *Trichogramma* spp. and demonstration by Lewis et al. (1982) of increased parasitization in small field plots by use of the host sex pheromone show the merit of these considerations.

Experimental Example

A recent discovery with the parasitoid *M. croceipes* opens a new avenue for potential application. Lewis and Tumlinson (1988) found that when contacting and antennating feces of their *Heliothis* larval host, the female links plant and other volatile odors to a host recognition kairomone in the feces and associatively learns to fly to those volatile odors in search of hosts. Actual contact with hosts and/or oviposition are not a necessary part of this process. We visualize from the findings that artificial fecal pellets containing the reinforcing host recognition kairomone and desired volatile odors might be applied to a crop at a sufficient density to reinforce search and thus retain the parasitoids in a habitat even at low host densities. Further, by incorporating volatiles in the formulations that are more prevalent in certain parts of the plant, the elicited search behavior can be concentrated on certain portions of the plants or in other ways directed as desired. It is expected that similar phenomena, and thus manipulation prospects, exist for other parasitoid species. The purpose of the experimental data here is to illustrate the encouraging prospects of such a package, together with prerelease conditioning of the parasitoid. Although raw frass was

used in the following experiment, synthetic formulations could be substituted if the results continue to be successful.

Procedures—Experiment I. The prospect of using semiochemicals to manipulate the foraging behavior of *M. croceipes* females was tested in small field plots using raw host feces as the treatment formulation. Under three environmental conditions of host–host feces densities, and three levels of prerelease experience, individual females were released and observed in small (2 m × 3 m) plots of soybean, *Glycine max* (L.) Merrill (arrayed 6 plants × 3 rows, 18 plants per plot). Behaviors such as flight, antennation, and oviposition were monitored until the parasitoid left the plot.

Plants within the plot were artificially infested with laboratory-reared third-instar *H. zea* and soybean-fed *H. zea* feces at various densities to simulate three different foraging conditions. Host sites were comprised of a single *H. zea* larva and a small amount (ca. 25 mg) of feces placed next to the larva. The host was affixed to the plant with a small insect pin (No. 000) pushed through the last larval segment. Feces-only sites were comprised of a small amount of soybean-fed *H. zea* feces. The three environmental conditions under which foraging behavior was studied were as follows: (1) low host density–low feces density (LL)—two host sites per row; (2) low host density–high feces density (LH)—two host sites per row, all remaining plants contained feces-only sites; and (3) high host density–high feces density (HH)—all plants contained host sites.

Because experience can have such a profound effect on the behavior of *M. croceipes*, we also studied the influence of prerelease experience on the performance of this parasitoid. The three types of experience we studied were as follows: (1) complete–female was given four encounters with the full host—plant complex, including feces (i.e., the same as the host site described above); (2) limited–female was allowed to antennate soybean-fed *H. zea* feces for 1 min; and (3) naive–female was given no experience with host or host feces. Parasitoids were given their experience in a large cage (45 × 47 × 34 cm) at the field edge immediately prior to their release in the plot. The insects were transported to the test plot in a 2-dram shell vial and released into the center of the plot by allowing them to walk out of the vial onto a nontarget leaf.

The behavior of each parasitoid was closely observed and recorded using The Observer event recording program on a TRS-80 model 100 portable computer (Tandy Corp.) A total of six parasitoids was observed for each of the nine combinations of experience level and environmental parameters. Data were analyzed using the GLM procedure (SAS Institute, 1985) and significant differences among means were determined by the Waller-Duncan K-ratio t test (Waller and Duncan, 1969).

Procedures—Experiment II. Although previous studies have shown that contact with feces alone is sufficient for *M. croceipes* females to learn and subsequently fly to host-associated odors (Drost et al., 1986, 1988; Lewis and

Tumlinson, 1988), we suspected that actual host contact along with the feces could affect what was learned and the stability of that learned behavior over time. Such an effect would be important to the design of formulation and application patterns of semiochemical–parasitoid manipulation systems.

The influence of preflight treatment on the flight response of *M. croceipes* females over time was studied in a flight tunnel. Three-day-old *M. croceipes* were given one of two pre-flight treatments: (1) antennation of cowpea (*Vigna unguiculata* L.) -fed *H. zea* feces for 30 sec, or (2) antennation of feces followed by an encounter and subsequent oviposition on a third-instar *H. zea*. Treated insects were then tested in the flight tunnel after one of seven time intervals: 1 min or 1, 3, 6, 12, 24, or 48 hr. Those insects tested after only 1 min were kept in a 2-dram shell vial until introduction into the flight tunnel. Those insects tested after 1 hr or more were held in Plexiglas cages (30 × 30 × 17 cm) under normal rearing conditions (28°C, 50–70% relative humidity, 16 hr photophase), and provided with food and water.

After the appropriate time interval, the response of the females was monitored in a Plexiglas flight tunnel. The test section of the tunnel was 2 m long with a 75 × 75-cm cross section. The flight tunnel provided laminar flow at 31 cm/sec. The parasitoids were released individually from 2-dram shell vials 120 cm downwind from the odor source. The odor source consisted of 40 ml of a hexane extract of cowpea-fed *H. zea* feces (=4 mg eq.). A successful flight was one in which the female exhibited a host-seeking flight response and landed on the target. Each female was given two chances, or two flights, to reach the target. For each experiment, six females from the same cage were randomly chosen and flown after a given time interval. For each time interval, the experiment was replicated 10 times on separate days for a total of 60 insects.

Results—Experiment I. Retention time in the experimental plots by *M. croceipes* females was highly dependent upon prerelease experience as well as the density of host–host feces in the foraging environment (Figure 3). Retention in the plot was highest for those insects given either a complete or a limited experience and released in an environment that contained a host site on each plant (HH). Conversely, retention times were lowest for naive insects, and times were not significantly different between the foraging environments.

Within each of the foraging environments, retention time in the plot was virtually identical for insects that were given either a complete or a limited experience. This indicates that the tendency to disperse, as seen in naive insects, was greatly reduced once the parasitoid gained some experience with either a host or host feces.

The parasitization rate also was influenced highly by experience level and host–host feces density within the plot (Figure 4). For insects that were given a complete experience, the parasitization rate increased steadily as the number of host–host feces sites increased from LL to HH. For those insects given a

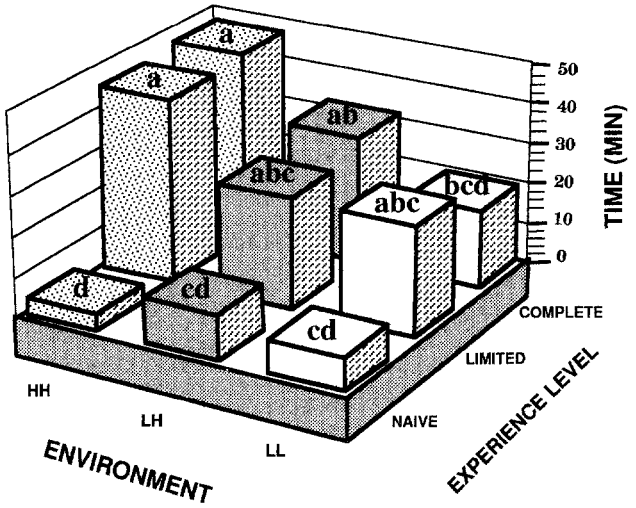


FIG. 3. Total time, in minutes, spent in small field plots of varying densities of host–host feces by female *Microplitis croceipes* given one of three different experiences. HH = high host, high feces density; LH = low host, high feces density; LL = low host, low feces density. Complete—female was given four encounters with the full host-plant complex, including feces; limited—female was allowed to antennate soybean-fed *H. zea* feces for 1 min; naive—female was given no experience with host or host feces. Bars topped by the same letter are not significantly different ($P = 0.05$; Waller-Duncan k-ratio t test).

limited experience, parasitization rates were only slightly increased as the number of host–host feces sites increased. No parasitization was achieved by naive insects.

Results—Experiment II. After a 1-min interval from treatment to flight, there was no significant difference in the flight response by *M. croceipes* females given either of the two preflight treatments (Figure 5). After this time, however, there was a much more dramatic decay in the response by those insects given contact with host feces only. After only 3 hr, flight responses in those insects reached their lowest point and leveled off at approximately 16–17%. At 24 hr, there was a slight increase in the percent of successful flight to 28% before falling again at 48 hr after treatment. Fluctuation in the response by insects exposed to feces only supports the argument. The response by females given oviposition experience in addition to a preflight exposure to feces, on the other hand, was consistently stronger and much more stable over time. Again, response leveled off 3 hr after treatment, but in this case remained at approximately 60%.

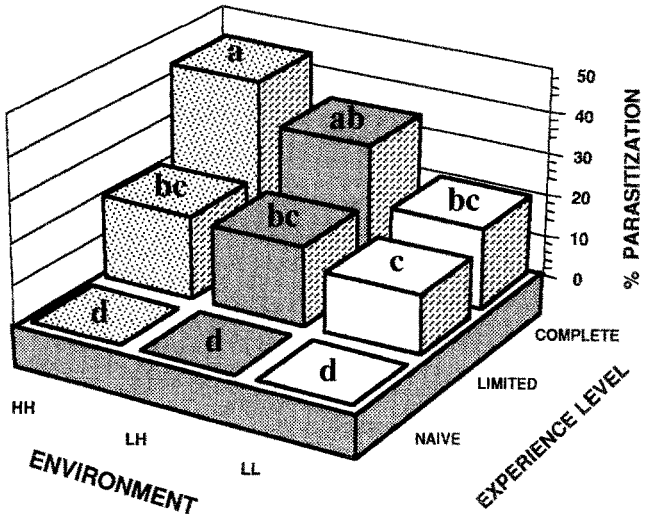


FIG. 4. Percent parasitization of third-instar *Heliothis zea* in small field plots by *Microplitis croceipes* given one of three different experiences under varying densities of host–host feces. See Figure 3 caption for full description of environment and experience level. Bars topped by the same letter are not significantly different ($P = 0.05$; Waller-Duncan k-ratio t test).

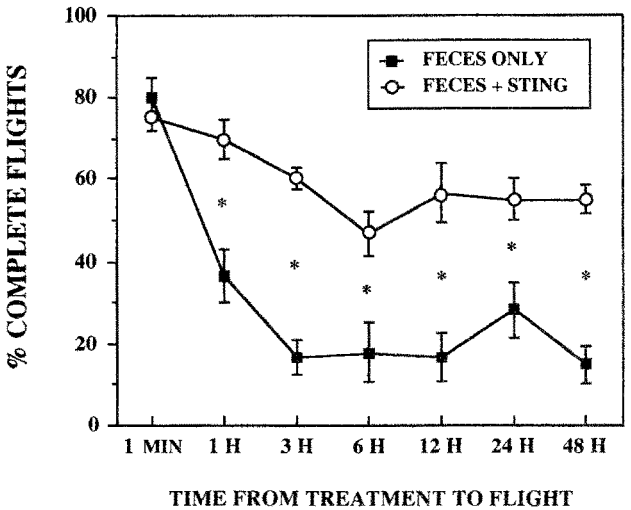


FIG. 5. Effect of preflight experience on the flight response to a volatile host-seeking stimulant at different time intervals by female *Microplitis croceipes* in a flight tunnel. Points are the means of 10 replications of six insects each; vertical bars represent SEM. Points with an asterisk between them are significantly different ($P < 0.05$) as determined by t test after angular transformation.

Discussion. The results presented here indicate that behavioral enhancement of *M. croceipes* is possible through the manipulation of both the parasitoid and its foraging environment. We have shown that an experienced insect loses its tendency to disperse and will actively search for hosts in an area for a much longer period of time than a naive insect. By manipulating the density of hosts and host products, foraging efficiency and parasitization rates were enhanced in small field plots. Further, by giving the parasitoid an oviposition experience, we demonstrated that flight response by *M. croceipes* to a volatile host-finding semiochemical is much more stable, with respect to time, than if the parasitoid was given an experience with host feces only. Other studies in our laboratory indicate that certain spatial information and odor preferences can be affected through oviposition experience (Wackers, personal communication). These factors will need to be better understood and properly incorporated into the design of future applied semiochemical–parasitoid manipulation systems.

The methods and results represent portions of various studies currently under way or recently completed and will be reported in greater detail in forthcoming publications.

SUMMARY AND CONCLUSION

The major role of semiochemicals in mediating the interactions of parasitoids with their hosts and habitats has been demonstrated for numerous parasitoids. To date, direct applications of those materials to enhance parasitoid performance has been limited to small-scale field plots. However, rapid advancements are being made relative to airborne chemicals and longer-range foraging behavior. Moreover, recent discoveries have shown that a combination of genetic diversity in parasitoid populations and phenotypic plasticity of individuals together with their physiological state often results in substantial variation in the preferred mediating chemicals and the nature of the response. We must understand these sources of variation and match them in accordance with the conditions of the environment in which the parasitoid must forage for hosts.

In the short term, this knowledge will allow us to define the genetic and phenotypic foraging profiles important to consistent and efficient parasitoid foraging behavior and to establish the proper propagation and release procedures and monitoring bioassays necessary for ensuring the appropriate behavioral and physiological qualities of the released organisms. In the longer term we envision technology for comprehensively manipulating the target pest–crop environment to provide foraging stimuli and other needs important for retention and efficiency by parasitoids.

Successful utilization of semiochemical–parasitoid systems requires inter-

vention and management from the parasitoid and foraging environment sides of the equation. Emerging technologies for such applications are encouraging.

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PLANT STRATEGIES OF MANIPULATING PREDATOR–PREY INTERACTIONS THROUGH ALLELOCHEMICALS: PROSPECTS FOR APPLICATION IN PEST CONTROL

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Abstract—To understand the role of allelochemicals in predator–prey interactions it is not sufficient to study the behavioral responses of predator and prey. One should elucidate the origin of the allelochemicals and be aware that it may be located at another trophic level. These aspects are reviewed for predator–prey interactions in general and illustrated in detail for interactions between predatory mites and herbivorous mites. In the latter system there is behavioral and chemical evidence for the involvement of the host plant in production of volatile allelochemicals upon damage by the herbivores with the consequence of attracting predators. These volatiles not only influence predator behavior, but also prey behavior and even the attractiveness of nearby plants to predators. Herbivorous mites disperse away from places with high concentrations of the volatiles, and undamaged plants attract more predators when previously exposed to volatiles from infested conspecific plants

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rather than from uninfested plants. The latter phenomenon may well be an example of plant-to-plant communication. The involvement of the host plant is probably not unique to the predator–herbivore–plant system under study. It may well be widespread since it makes sense from an evolutionary point of view. If so, prospects for application in pest control are wide open. These are discussed, and it is concluded that crop protection in the future should include tactics whereby man becomes an ally to plants in their strategies to manipulate predator–prey interactions through allelochemicals.

Key Words—Predator, prey, host plant, tritrophic interactions, plant defense, allelochemicals, kairomone, pest control, behavior, chemical identification.

INTRODUCTION

Entomophagous arthropods use a wealth of chemical information when searching for hosts or prey (Nordlund et al., 1981a; Bell and Cardé, 1984; Haynes and Birch, 1985; Barbosa and Letourneau, 1988). Most of the published studies concern parasitoids; predators have been much less studied. Recently, however, data on the role of allelochemicals in predator foraging have increased for some groups such as predatory mites, bark beetle predators, and coccinellids (Billings and Cameron, 1984; Heuer and Vité, 1984; Payne et al., 1984; Sabelis and Dicke, 1985; Baisier et al., 1988; Dicke and Sabelis, 1988b; Hammond, 1988). This paper reviews that progress and presents new data. This is done with the aim of finding prospects for application of allelochemicals in pest control.

The focal points of this paper are the effect of allelochemicals on predator behavior and the origin and chemical nature of the allelochemicals. Application of allelochemicals in pest control is impossible without knowledge of the behavior induced by the chemicals (Lewis et al., 1975a,b, 1979; Gross, 1981). In addition, to manipulate predator–prey interactions with allelochemicals it is necessary to know the origin of the compounds, and thus the organism(s) that control their production. Finally, elucidation of the chemical structure is indispensable for synthesis of the allelochemicals.

Much of the research on allelochemicals affecting behavior of natural enemies of herbivores has been done in a bitrophic (predator–prey; parasitoid–host) context (Nordlund et al., 1981a; Bell and Cardé, 1984; Haynes and Birch, 1985), but since the papers by Price et al. (1980) and Price (1981) it is clear that plants are often an essential factor, either passive or active, affecting predator–prey interactions. Interactions mediated by allelochemicals are no exception and recent data, presented in this paper, reveal interesting plant activities such as beating the alarm or overhearing. Because plant activities seriously affect predator–prey interactions, it is essential to view the three focal points mentioned above in a tritrophic context.

ALLELOCHEMICAL TERMINOLOGY

Nordlund and Lewis (1976) presented a semiochemical terminology that was developed at a time when interactions were almost exclusively studied in a bitrophic context. Various semiochemical categories have been distinguished. They are based on: (1) whether or not the interaction is between conspecific individuals (pheromones vs. allelochemicals), (2) which costs and benefits fall to each of the two interacting organisms, and (3) which organism is the producer and which the receiver. Since tritrophic systems are of much greater complexity, several difficulties in using the terms have been encountered. In a reevaluation of this terminology, Dicke and Sabelis (1988a) argued that the producer criterion should be dropped. They presented several examples showing that organisms once thought to be producers later on appeared to be not the producers themselves, but rather to be associated with other organisms (e.g., microorganisms) that were responsible for allelochemical production or for induction of production in another organism. By taking the producer criterion strictly, terminology would exclude an important class of ecologically significant interactions (Dicke and Sabelis, 1988a). The terms in this paper are used according to this updated terminology: for any allelochemical the term used is based on the cost–benefit analysis for the two interacting organisms under consideration (see Table 1 for definitions).

In addition, Dicke and Sabelis (1988a) emphasized the special status of information-conveying chemicals: this category differs from toxins and nutrients in that they are not detrimental or beneficial themselves, but may be through the response they elicit. Information-conveying chemicals were termed infochemicals (Table 1) and constitute a subcategory of semiochemicals.

ROLE OF ALLELOCHEMICALS IN FORAGING BEHAVIOR OF PREDATORY ARTHROPODS

Predator foraging consists of a series of behaviors that are affected by information about the surroundings. Chemicals are among the main information-conveying agents available to predatory arthropods. They play an essential role in almost all stages of prey searching and prey selection. Predators may exploit chemical information in two main foraging strategies:

1. The predator lies in wait and attracts prey. This phenomenon has been studied in depth for bolas spiders. These predators produce allomones that are mimics of female moth sex pheromones and thus attract male moths of a restricted number of species that are caught by means of a sticky ball on the end of a silken thread (Eberhard, 1977; Stowe et al., 1987; Yeargan, 1988).

TABLE 1. INFOCHEMICAL TERMINOLOGY (DICKE AND SABELIS, 1988a)

Infochemical: A chemical that, in the natural context, conveys information in an interaction between two individuals, evoking in the receiver a behavioral or physiological response.

Pheromone: An infochemical that mediates an interaction between organisms of the same species whereby the benefit is to the origin-related organism [(+, −) pheromone], to the receiver [(−, +) pheromone], or to both [(+, +) pheromone].

Allelochemical: An infochemical that mediates an interaction between two individuals that belong to different species.

Allomone: An allelochemical that is pertinent to the biology of an organism (organism 1) and that, when it contacts an individual of another species (organism 2), evokes in the receiver a behavioral or physiological response that is adaptively favorable to organism 1, but not to organism 2.

Kairomone: An allelochemical that is pertinent to the biology of an organism (organism 1) and that, when it contacts an individual of another species (organism 2), evokes in the receiver a behavioral or physiological response that is adaptively favorable to organism 2, but not to organism 1.

Synomone: An allelochemical that is pertinent to the biology of an organism (organism 1) and that, when it contacts an individual of another species (organism 2), evokes in the receiver a behavioral or physiological response that is adaptively favorable to both organism 1 and 2.

2. The predator actively searches for prey. This strategy is the more common one, and allelochemicals play a role at several stages:

(a) In an initial phase of prey searching, predators may use plant volatiles as synomones in the selection of a suitable foraging habitat. It may be presumed that these synomones affect in-flight behavior of predators, but most behavioral assays have tested the animals in the ambulatory phase (Greany and Hagen, 1981; Vinson, 1981; Dwumfour, 1987; Reid and Lampman, 1989).

(b) Volatile prey-related chemicals may be used as kairomones in an early stage of prey searching (Vité and Williamson, 1970; Mpakagiannis, 1982; Aldrich et al., 1986; Sabelis and Afman, 1984; Sabelis and Dicke, 1985; Sabelis and Janssen, 1990). In-flight behavior may be influenced (Vité and Williamson, 1970), but alternatively, upon landing, the decision to stay or to continue aerial dispersal may be affected by volatile kairomones (Sabelis and Afman, 1984; Sabelis and Janssen, 1990). These compounds obviously provide more information about prey presence than plant volatiles and may function in distant prey selection (Sabelis and Van de Baan, 1983; Dicke et al., 1988).

(c) Once in a prey patch, predators may be arrested by volatile or nonvolatile kairomones (Hislop and Prokopy, 1981; Hoy and Smilanick, 1981; Evans and Dixon, 1986; Sabelis et al., 1984b; De Moraes and McMurtry, 1985) through kinetic and tactic responses of the predator at the patch edge (Hislop and Prokopy, 1981; Sabelis et al., 1984b).

(d) Due to predator activity, prey density may decline locally and at some moment a predator decides to leave the patch to search for other, more rewarding, patches. Kairomones affect this decision (Sabelis and Afman, 1984; Sabelis and Janssen, 1990).

(e) Prey searching is not the only process affected by allelochemicals. For example, some predators use these cues to select oviposition sites (Hagen 1986; Evans and Dixon, 1986; Baisier et al., 1988). This is especially relevant when the larva is predacious and the adult is not, or consumes a different prey located elsewhere in the environment.

In conclusion, it is clear that allelochemicals affect many stages of predator foraging behavior through a variety of behavioral responses. As a consequence, thorough behavioral analysis is indispensable for drawing conclusions on the role of allelochemicals in predator foraging strategies and thus for possibilities to employ such chemicals in pest control.

ORIGIN OF ALLELOCHEMICALS INVOLVED IN PREDATOR FORAGING BEHAVIOR

Under the assumption that foraging behavior has a genetic basis, natural selection is expected to favor those predator genotypes that possess a foraging strategy that results in maximum reproductive success. Thus, the source of the allelochemicals used by predators may be expected to vary, under the condition that the chemicals involved increase the rate of prey finding. Indeed, foraging predators exploit allelochemicals from their prey, or prey activities, but also from the host plant, or from other organisms associated with the prey, such as microorganisms. Many examples exist for predators using prey-produced allelochemicals (for review, see Greany and Hagen, 1981), but fewer have been recorded for allelochemicals produced by the prey's host plant (Vinson, 1981; Nordlund et al., 1988) or associated microorganisms (Dicke, 1988a). However, this is presumably due to restricted efforts made to find the exact source of the allelochemical (for review, see Dicke and Sabelis, 1988a).

Elucidation of the origin of allelochemicals that affect predator foraging behavior is not only interesting from a theoretical point of view (Dicke and Sabelis, 1988a) but may also provide application possibilities. Identification of the origin of the allelochemical provides prospects for investigations aiming at finding ways to manipulate release rates. This will be illustrated in this paper.

CHEMICAL NATURE OF ALLELOCHEMICALS AFFECTING PREDATOR-PREY INTERACTIONS

Many allelochemicals that function in predator–prey interactions originally served as pheromones for the prey individuals: predators illegitimately use the chemicals either as receiver or as emitter. Thus, elucidation of the chemical

structures of allelochemicals has benefitted in many cases from studies on pheromone structures. This is most evident for predators of bark beetles. The wealth of knowledge on bark beetle pheromones (Wood, 1982; Birch, 1984; Borden, 1984) has caused a boom in chemical knowledge of kairomones that attract their predators (Vité and Williamson, 1970; Billings and Cameron, 1984; Chatelain and Schenk, 1984; Heuer and Vité, 1984; Payne et al., 1984). In addition, several tree terpenes whose release is increased by herbivore damage, and that function as synomones in plant–predator interactions, have been identified (Fitzgerald and Nagel, 1972; Baisier et al., 1988). Similarly, Aldrich et al. (1986) identified a pheromone of a stink bug and showed that it attracts its predator, the eastern yellowjacket. After good indications for the production of moth sex-pheromone components by bolas spiders to function as allomones in attracting male moths as prey (Eberhard, 1977), chemical investigations confirmed this (Stowe et al., 1987).

Pheromone identification has not always given the impetus to research aimed at structure identification. There are cases where the impetus came from investigations on predator–prey interactions. Lacewing adults (*Chrysopa carnea*), which feed on honeydew, are attracted to indole acetaldehyde, a breakdown product of the honeydew component tryptophan (Hagen et al., 1976; Van Emden and Hagen, 1976). The offspring produced on honeydew-contaminated plants feed on the aphids that deposited the honeydew. *Chrysopa* adults are also attracted to plant compounds such as caryophyllene, which presumably represents habitat location (Flint et al., 1979). Chemical analysis of volatile kairomones that attract predatory mites to prey-infested plants not only resulted in identification of kairomone structures but also revealed pheromonal and synomonal functions of these chemicals in a tritrophic context (Dicke, 1988c; Dicke et al., 1990a). Instead of being a consequence of pheromone identification, elucidation of allelochemical structures in this case was the first step in a process of unravelling interactions mediated by infochemicals, both pheromones and allelochemicals. Details of this example are given below (section "Allelochemicals in a tritrophic system consisting of predatory mites, spider mites, and their host plants").

FUNCTION OF ALLELOCHEMICALS IN A MULTITROPHIC CONTEXT

Once an infochemical is released, any organism that encounters it is a potential user and thus every infochemical may affect several interactions in a multitrophic system (Price et al., 1980; Price, 1981). Consequently, when considering the function of an allelochemical, care must be taken to investigate all components of the system. For instance, if a predator uses a volatile produced by its herbivorous prey as a kairomone in prey location, it is important to investigate what the function of this compound is to the prey (e.g., Bakke and

Kvamme, 1981; Payne et al., 1984; Dicke, 1986). Moreover, it appears to be also important to investigate the function of this infochemical to potential host plants of the herbivore: they may use the information to take defensive action. This was demonstrated for a system of cotton plants and a pathogenic fungus (Zeringue, 1987). However, the response of host plants to chemical information has as yet received limited attention (Baldwin and Schultz, 1983; Rhoades, 1985a,b; Bruin et al., 1990).

ALLELOCHEMICALS AFFECTING FORAGING BEHAVIOR OF NATURAL ENEMIES AND APPLICATION IN PEST CONTROL

Research on allelochemicals and natural enemies of herbivores has expanded enormously since the 1970s. This initiated many ideas about application of these infochemicals in pest control, such as: (1) enhancing searching efficiency of natural enemies (Lewis et al., 1975a,b, 1979; Gross, 1981); (2) bringing the natural enemies in a specific search mode (Gross, 1981; Vet, 1988); (3) making novel or artificial host–prey species acceptable in a mass rearing program (Vinson, 1986); (4) using the response to the allelochemical as a criterion in the selection of natural enemies for biological control (Janssen et al., 1990) or (5) as a criterion in quality control (Dicke et al., 1989c; Noldus, 1989); and (6) breeding plant cultivars that have an increased emission rate of natural-enemy-attracting synomones (Nordlund et al., 1981b, 1988).

The idea of enhancing search efficiency through application of allelochemicals has been seriously tested in laboratory and field situations with *Trichogramma* spp., egg parasitoids of moth species such as *Heliothis*. Although this work concerns parasitoids rather than predators, it is referred to in this paper because it is the best investigated system with respect to application of allelochemicals in pest control. The knowledge gained is thought to have profound implications for predator–prey interactions as well.

Initial tests showed that enhanced parasitization rates may be obtained by spraying an extract of moth scales or synthetic kairomone identified in these scales (Lewis et al., 1975a,b). However, subsequent testing revealed that, when using a blanket spray, this result could only be obtained at high host densities, not at natural densities. At low or intermediate densities, parasitization rates could be increased by applying kairomone or artificial moth scales (diatomaceous particles impregnated with kairomone) around the host eggs (Lewis et al., 1979; Gross, 1981). To understand these results, knowledge of the behavioral response induced in the parasitoid upon contacting kairomone is indispensable. Moth scales are present around oviposition sites and represent moth activity on a larger area than the egg alone. Upon contacting (kairomone in) moth scales, the parasitoids increase searching activities locally and are arrested (Beevers et al., 1981; Noldus and Van Lenteren, 1985; Gardner and Van Len-

teren, 1986). Thus, when kairomone is applied on leaves without hosts, parasitoids waste time, which results in a reduction in the parasitization rate. At low ratios of host-containing leaves to leaves without hosts, this effect is most pronounced. At extremely high densities, there is a net positive effect, because there are no host-deficient leaves (Lewis et al., 1975a,b; 1979). This research led to the conclusion that kairomone distribution in combination with host distribution decisively affected the outcome of the experiments as a result of the behavioral response of the parasitoids. Increasing the parasitization rate under field conditions by increasing kairomone concentration at or in the neighborhood of oviposition sites is obviously not a practical solution for pest management, however. This research project demonstrated that knowledge of the foraging behavior is essential for successful employment of the allelochemical in pest control. Similar conclusions were obtained by Chiri and Legner (1983) in research on an egg-larval parasitoid of the pink bollworm.

The idea of applying allelochemicals in pest control through plant breeding has received increased interest in recent years because of biotechnological developments and their effects on plant breeding (Nordlund et al., 1988; Dicke et al., 1989a). Recent investigations of a tritrophic system consisting of predatory mites, spider mites, and their host plants indicate that plant breeding may solve problems about kairomone distribution, such as encountered for the *Heliothis–Trichogramma* system. This will be the subject of the remainder of this paper.

ALLELOCHEMICALS IN A TRITROPHIC SYSTEM CONSISTING OF PREDATORY MITES, SPIDER MITES, AND THEIR HOST PLANTS

Spider mites are polyphagous herbivores that reach pest status in many agricultural crops (see Helle and Sabelis, 1985a, for review). They insert their stylets in the leaves, inject saliva, and ingest parenchymous cell contents (Tomczyk and Kropczynska, 1985). An adult female gives rise to a colony from which daughters disperse upon reaching adulthood to settle nearby and initiate new colonies. Spider mites are particularly ravenous herbivores, overexploiting their food source in the absence of predators. However, local populations are decimated if discovered by predators such as predatory mites (see Helle and Sabelis, 1985b, for review). Allelochemicals play an essential role in this process.

Allelochemicals and Predatory-Mite Behavior

Allelochemicals affect prey-searching and prey-selection behavior of predatory mites in several ways: (1) Predatory mites disperse on wind currents and probably exert little control on where they land. Thus, the chances of landing

in a spider-mite colony or on a spider-mite-infested plant are small. However, after landing, volatile kairomones are used in making foraging decisions such as whether to stay or to take off again, and where or how long to search (Sabelis and Afman, 1984; Sabelis and Dicke 1985; Sabelis and Janssen, 1990). The response to these volatile kairomones depends on quantitative and qualitative food history of the predatory mites (Sabelis and Van de Baan, 1983; Dicke et al., 1986, 1989c). (2) Once predatory mites are in a prey patch [a group of leaves infested by prey (cf. Sabelis, 1981; Sabelis and Dicke, 1985)], their behavior is affected by volatile and nonvolatile kairomones. These chemicals induce kinetic and tactic responses in the patch and at the patch edge (Hislop and Prokopy, 1981; Sabelis et al., 1984a; Sabelis and Dicke, 1985). As a result the predators stay in the patch as long as prey-related allelochemicals are present. (3) Predatory mites distinguish between volatile kairomones related to different prey species: they have kairomone preferences (Sabelis and Van de Baan, 1983; Dicke and Groeneveld, 1986; Dicke, 1988b; Dicke et al., 1988) that correlate to prey preferences as assessed in predation analyses under laboratory and field conditions (Dicke and De Jong, 1988; Dicke et al., 1988, 1989b). Thus, distant information about prey availability initiates prey selection decisions similar to those made in the prey patch.

Source of Volatile Allelochemical Affecting Predatory-Mite Behavior

Most research has been conducted on a system consisting of Lima bean plants (*Phaseolus lunatus*), the spider mite *Tetranychus urticae*, and the predatory mite *Phytoseiulus persimilis*. These predatory mites distinguish between *T. urticae*-infested and uninfested Lima bean plants by olfaction. The volatile kairomone involved in this predator–prey interaction is mainly emitted from the leaves after infestation. Upon removal of spider mites and their visible products, previously infested leaves remained attractive to predatory mites for several hours, whereas the spider mites removed from the leaves were not attractive (Sabelis and Van de Baan 1983, Sabelis et al. 1984a). Several attempts have been made to gain more detailed knowledge of the origin of the kairomone by fragmentation of the spider mite colony or by extracting the leaf surface after spider-mite feeding had occurred (Sabelis et al., 1984a). The results show that all feeding spider-mite stages contributed to kairomone production and that the infested leaf was the main kairomone source. Very little kairomone activity was present in feces or in extracts of spider mites. No kairomone activity could be demonstrated in plants treated in several ways, unless spider-mite feeding had recently occurred. Although this investigation did not result in a final elucidation of the origin of the kairomone, it indicated that both spider mite and host plant are involved in its production. Note that we classify the allelochemical as a kairomone in this discussion as long as we deal with predator–prey interactions; in plant-predator interactions it is a synomone (Table 1).

Some further evidence for involvement of plant and spider mite in the production of kairomone is the following. Many different plant-spider mite combinations emit a volatile kairomone (Dicke and Sabelis, 1988b), and predatory mites distinguish between them (Sabelis and Van de Baan, 1983; Dicke and Groeneveld, 1986; Dicke et al., 1986; Dicke, 1988b). The volatile kairomone is spider-mite species specific: predatory mites distinguish between plants infested by different spider-mite species (Sabelis and Van de Baan, 1983). Moreover, recent experiments show that the kairomone is also plant species specific: *Phytoseiulus persimilis* females distinguish between *T. urticae*-infested Lima bean leaves and *T. urticae*-infested cucumber leaves. Predators from a stock culture on *T. urticae* on Lima bean leaves were transferred to either *T. urticae*-infested Lima bean leaves or *T. urticae*-infested cucumber leaves. After seven days, the predators were offered a choice between infested Lima bean leaves and infested cucumber leaves in a Y-tube olfactometer. The predators preferred the olfactometer arm with the odor source that they had experienced prior to testing (Figure 1). Because kairomone preference depends on predator experience, the discrimination is the result of a qualitative rather than a quantitative difference in kairomone (Dicke et al., 1990b).

Previously infested leaves are still highly attractive to predatory mites after removal of spider mites and their visible products. Thus, we investigated whether spider mites deposit the kairomone in invisible form on the leaf surface. For this, we studied whether kairomone activity on a spider-mite-infested plant is restricted to the infested leaves. Lima bean plants that had three or four leaves were infested on one of the primary leaves. Spider mites were prevented from leaving the leaf by applying Tanglefoot sticky material to the petiole. After six days at 25°C, uninfested leaves of infested plants were tested in a Y-tube olfactometer (see Sabelis and Van de Baan, 1983, for experimental setup) versus uninfested leaves of uninfested plants (equal leaf weights, in the range of 10–18 g, in both olfactometer arms). Uninfested plants were taken from the same batch as the plants that had been partly infested, and they had also been kept at 25°C for six days prior to the experiment. This was done in the same climate room as used for partly infested plants, but the air current in the climate room was such that uninfested plants did not receive air that had passed over partly infested plants. *Phytoseiulus persimilis* females preferred the odor of uninfested leaves of infested plants over the odor of uninfested leaves of uninfested plants (Figure 2A). This may be explained by either production of an allelochemical in uninfested leaves of infested plants or by adsorption and reemission of an allelochemical that was produced at the site of spider-mite infestation. To exclude adsorption, we carried out a similar experiment in which the infested leaves were enveloped in a Petri dish through which an airstream was generated to remove all emitted volatiles. In the Y-tube olfactometer experiment, the predator distribution was similar to that in the previous experiment (Figure 2B).

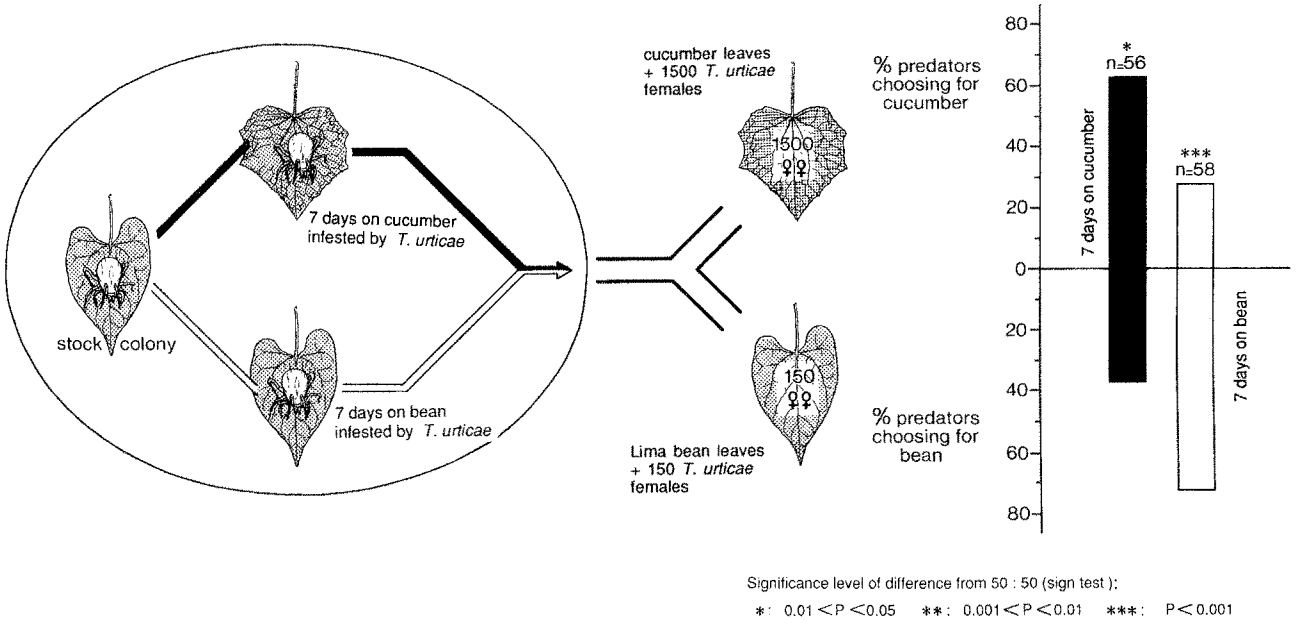


FIG. 1. Response of satiated *P. persimilis* females in a Y-tube olfactometer when offered *T. urticae*-infested Lima bean leaves vs. *T. urticae*-infested cucumber leaves. The ratio of spider-mite numbers of 1 : 10 was determined from preliminary experiments: the responses to each odor source were such that changes in preference were best detectable; at lower or higher ratios one of the odor sources evoked such a strong response that changes in preference were more difficult to detect (J. Takabayashi and M. Dicke, unpublished data). n: number of predators. Figure after data in Dicke et al. (1990b).

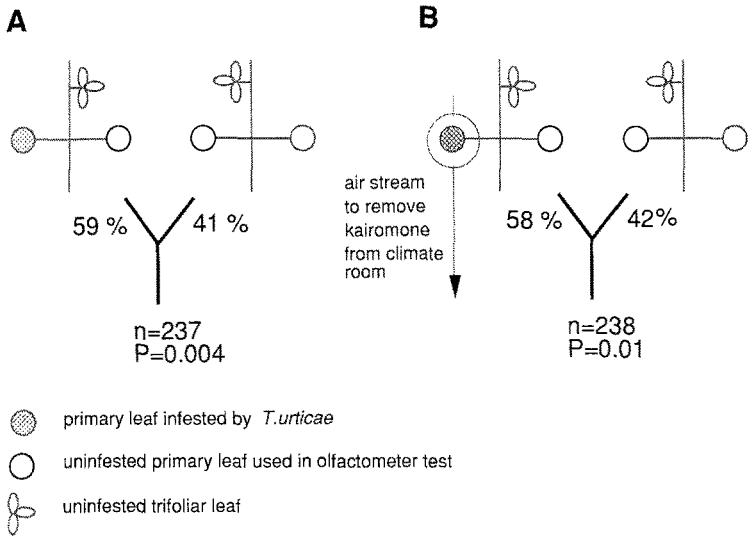


FIG. 2. Response of satiated *P. persimilis* females in a Y-tube olfactometer when offered uninfested leaves from infested Lima bean plants vs. uninfested leaves from uninfested Lima bean plants (equal leaf weights, within the range of 10–18 g, in both olfactometer arms). All plants were placed in a climate room (25°C) during six days, before uninfested primary leaves were picked to be used in the olfactometer. For further details see text. (A) Volatiles emitted from infested leaves may adsorb to uninfested leaves; (B) volatiles emitted from infested leaves are led to the vacuum system of the building to prevent adsorption to uninfested leaves. n: number of predators tested, P: critical level determined with sign test for differences from 50:50 distribution of predators over the two arms.

This indicates that an attractant is produced in uninfested leaves of infested plants. This is the first evidence that production of predator attractants and spider mite damage can be locally separated. Whether the same predator attractants are produced in spider-mite-infested leaves and in uninfested leaves of infested plants remains to be investigated.

Chemical Identification of the Kairomone

Chemical investigations (Dicke, 1988c; Dicke et al., 1990a) revealed that large amounts of volatiles are emitted from artificially damaged or *T. urticae*-infested Lima bean plants. Identification of these compounds revealed only chemicals that belong to classes that are well-known from the plant kingdom. Four of the compounds attracted *P. persimilis* females. These were identified in the headspace of *T. urticae*-infested Lima bean plants but were not found in

the headspace of undamaged or artificially damaged plants. These kairomone components are the terpenes linalool and (*E*)-β-ocimene, the methylene terpene 4,8-dimethyl-1,3(*E*),7-nonatriene, and the phenolic compound methyl salicylate (Figure 3). These classes of compounds are known to be produced by plants, but not by animals. Linalool, (*E*)-β-ocimene, and methyl salicylate are known from many plant species. However, the methylene terpene deserves special attention, because it has been identified only recently in plants (Maurer et al., 1986; Kaiser, 1987). It was not detected in steam distillates of uninfested Lima bean plants (H. van Bokhoven and T.A. van Beek, unpublished data).

While none of the four kairomone components was found in the volatile blend emitted by undamaged or artificially damaged Lima bean plants [many analyses during several years; (Dicke et al., 1990a)] and undamaged plants were not attractive to *P. persimilis* (Sabelis and Van de Baan, 1983), most recent analyses consistently revealed small amounts of the four predator-attracting compounds in the headspace of undamaged or artificially damaged Lima bean plants. The amounts are of the same order of magnitude for clean and artificially damaged plants, but much lower (up to 200 times) than in spider-mite-infested plants (Takabayashi, Dicke and Posthumus, in preparation). Also, a slight attractiveness of undamaged Lima bean to *P. persimilis* is recorded in

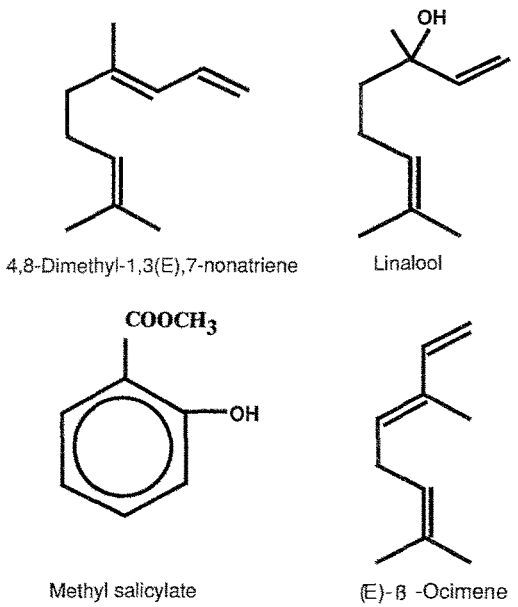


FIG. 3. Chemical structure of kairomone components identified in headspace of Lima bean plants infested by *T. urticae*.

recent experiments (Takabayashi et al., 1990, and in preparation). The plants used were never damaged by spider mites and have never been in the same room as spider-mite-infested plants. Thus, these recent data show that spider-mite damage is not necessary for the production of the allelochemicals, but that spider-mite damage, in contrast to artificial damage, increases the emission enormously. The discrepancy between recent data and those by Dicke et al. (1990a) for undamaged and artificially damaged plants is under investigation.

Recently, the headspace of *T. urticae*-infested cucumber plants (*Cucumis sativus* cv. Santo, F1) was analyzed (Takabayashi, Dicke, Posthumus, Van Beek, and De Bie, in preparation). Again, all compounds found were known to be produced by plants, and not by animals. The blend of volatiles emitted is different from the one emitted from *T. urticae*-infested Lima bean plants (Table 2). Of the four kairomone components emitted from infested Lima bean plants, only (*E*)-β-ocimene and 4,8-dimethyl-1,3(*E*),7-nonatriene were found in infested cucumber plants. In addition, some compounds that were discovered in the headspace of infested cucumber plants had not been found in infested Lima bean plants. Among these were two oxime ethers and two nitriles. The chemicals found in the headspace of infested cucumber plants are currently tested for attractiveness to predatory mites.

Although the chemical evidence corroborates the biological evidence that the plant is involved in kairomone production, it remains unknown how this is accomplished.

Is the Kairomone Important in Extermination of Spider Mites by Predatory Mites?

Simulation models of the local population dynamics of spider-mites (*T. urticae*) and predatory mites (*P. persimilis*) gave exceedingly bad predictions when the predators were assumed to search at random as they do in prey-infested leaf areas. However, if the predators, upon leaving the prey-infested leaf area, are assumed to return immediately, the simulation models gave reasonably good predictions (Sabelis and Van der Meer, 1986). Behavioral studies have demonstrated that the volatile–kairomone gradient present at the edge of the patch affects such returning behavior (Sabelis et al., 1984b) and that, even when starved, the predatory mite *P. persimilis* did not take off on wind currents as long as kairomone was present (Sabelis and Afman, 1984; Sabelis and Janssen, 1990). These investigations indicate that the volatile kairomone is essential in spider-mite extermination.

Effects of Predator-Attracting Allelochemical in Other Interactions in the Tritrophic System

So far, tritrophic aspects only referred to predatory mites as the organisms responding to the allelochemical of spider-mite-infested plants, but spider mites

TABLE 2. IDENTIFICATION OF CHEMICALS IN HEADSPACE OF LIMA BEAN PLANTS AND CUCUMBER PLANTS INFESTED BY SPIDER MITE *T. urticae*[a]

Compound	Relative percentage in headspace of *T. urticae* infested	
	Lima bean leaves	Cucumber leaves
Aldehydes		
2-Hexenal	<1	
Ketones		
2-Butanone	3–7	
3-Pentanone	<1	
Alcohols		
2-Methyl-propan-1-ol	<1	
1-Butanol	<1	
1-Penten-3-ol	<1	
(*Z*)-3-Hexen-1-ol	1–10	<1
1-Octen-3-ol	1–10	
1-Hexanol	<1	<1
2-Ethyl-1-hexanol		2–10
2-Octene-1-ol		<1
Esters		
(*Z*)-3-Hexen-1-yl acetate	20–40	1–10
(*Z*)-3-Hexen-1-yl butyrate	<1	<1
Methyl salicylate	1–5	
Terpenoids		
(*E*)-β-Ocimene	10–30	20–50
(*Z*)-β-Ocimene	<1	<1
4,8-Dimethyl-1,3(*E*),7-nonatriene	10–30	20–50
Linalool	1	
4,8,12-Trimethyl-1,3(*E*),7(*E*),11-tridecatetraene	5–15	1–5
Limonene	tr	1–10
(*E*,*E*)-α-Farnesene		1–5
Nitriles		
2-Methylbutanenitrile		<1
3-Methylbutanenitrile		1–5
Oximes		
2-Methylbutanal, *O*-methyloxime		<1
3-Methylbutanal, *O*-methyloxime		5–15

[a]For methods see Dicke et al. (1990a).

respond as well. The spider mite *T. urticae* is attracted by a volatile kairomone of undamaged Lima bean plants, but the mites disperse from an odor plume of Lima bean plants infested by conspecifics (Dicke, 1986). In the context of intraspecific spider-mite interactions the volatile infochemical emitted from *T. urticae*-infested plants is a pheromone; in this case, a (+, +)-dispersing pher-

omone (Table 1) (Dicke and Sabelis, 1988a). The behavioral reaction depends on the ratio of pheromone to kairomone. At a low ratio (low degree of infestation), the mites are attracted; at a high ratio (high degree of infestation), they are repelled. From biological evidence alone, Dicke (1986) argued that the pheromone shares components with the predator–prey kairomone. Indeed, from the combined data of Dabrowski and Rodriguez (1971), Dicke (1986), and Dicke et al. (1990a), it can be deduced that at least one component of the spider-mite pheromone is also a component of the predator–prey kairomone linalool. Methyl salicylate is not, while no data are available for the response of *T. urticae* to (*E*)-β-ocimene and 4,8-dimethyl-1,3(*E*),7-nonatriene.

Thus, several interactions in this system are mediated by volatile infochemicals whose release is induced by spider-mite feeding activities. An important question is: do predatory mites "spy" on spider-mite communication or do spider mites leave as soon as the plant's "cry for help" gets too loud? This is essentially a question about who controls production and/or release of the chemicals.

1. The spider mites might control production of the infochemical to inform conspecifics about local density and thus about food quantity and prospects for competition, but the spider mites would not need a volatile compound for this because information can also be conveyed by nonvolatile chemicals or by contacts between individuals, without the costs of volatiles or the associated risks of attracting predatory mites.

2. The plant might control production of volatiles to recruit predatory mites as "bodyguards." The volatile nature of the chemicals is then indispensable for fast spread of the advertisement: success of induced indirect defense (cf. Price et al., 1980, Dicke and Sabelis, 1988b) depends heavily on rapidly recruited defenders. In this case the spider mites would do best by using the volatiles as a dispersing pheromone. It seems unlikely that the mites could avoid the feeding-dependent release of the infochemical, unless they can make their feeding unrecognizable to the plant.

These considerations suggest that the volatile predator-attracting allelochemical primarily functions in plant-predatory mite interactions and that the spider-mite response is secondary.

New View on the Role of the Plant in the Tritrophic System of Predatory Mites, Spider Mites, and Their Host Plants

The role of the plant in this system appears to have unprecedented characteristics, and most recent data even add new aspects. It is important for plants to attract predatory mites at an early stage of spider-mite infestation. Kairomone emission occurs to a large extent upon spider-mite damage, but the emission of predator attractant is not restricted to the damaged tissue (Figure 2). This made

us wonder whether uninfested plants that are in the neighborhood of infested plants produce (increased amounts of) predator attractants. Such still-uninfested plants run high risks of becoming infested and might take preventive action. To investigate this, we used a three-compartment wind tunnel (Figure 4). Uninfested plants of one batch were placed in all three compartments. Spider-mites were released on the plants in compartment 2. Plants in compartments 1 and 3 remained uninfested. After several days the uninfested plants of compartment 1 and 3 were transferred to a Y-tube olfactometer setup to investigate the response of the predatory mite *P. persimilis*. After four to five days of incubation in the wind tunnel, the plants of compartment 3 (that had infested plants upwind) were more attractive than plants from compartment 1 (that had no plants upwind). This was done with Lima bean plants and cotton plants (Figure 5). When the setup was slightly modified so that the control plants had received the odor of uninfested plants, similar results were obtained, but the incubation time needed was somewhat longer (Figure 6).

Preliminary chemical investigations have revealed that the headspace of Lima bean plants from compartment B3 (Figure 6) contains relatively large amounts of (*E*)-β-ocimene and (to a lesser extent) 4,8-dimethyl-1,3(*E*),7-nonatriene: ca. 17 and 5 times more than in undamaged plants that had not been in the neighborhood of infested plants. Thus, the composition of the blend emitted by plants from compartment B3 is completely different from the blend of control plants. For instance, in analyses of the headspace of undamaged plants that never experienced *T. urticae* infestations, peak areas of (*E*)-β-ocimene and 4,8-dimethyl-1,3(*E*),7-nonatriene are smaller than or equivalent to that of (Z)-3-hexen-1-yl acetate, but in the headspace of plants from compartment B3, peak areas are up to five times [4,8-dimethyl-1,3(*E*),7-nonatriene] and up to 26 times [(*E*)-β-ocimene] larger than that of (Z)-3-hexen-1-yl acetate! These data may be explained by adsorption of 4,8-dimethyl-1,3(*E*),7-nonatriene and (*E*)-β-ocimene emitted from plants in compartment B2 on plants in compartment B3, but the amounts released from plants in compartment B3 are rather

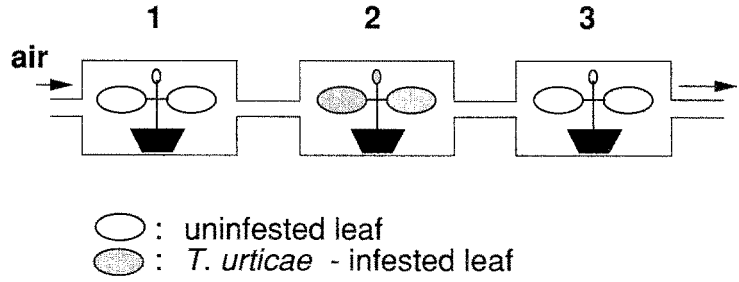


FIG. 4. Three-compartment wind-tunnel.

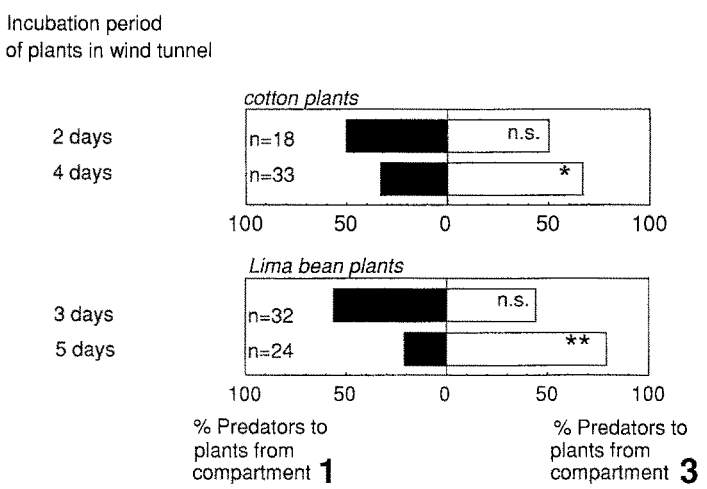


FIG. 5. Response of satiated *P. persimilis* females in a Y-tube olfactometer to plants from compartment 1 vs. plants from compartment 3 of the three-compartment wind tunnel. $^{**}P \leq 0.01$; $^{*}0.01 < P \leq 0.05$; n.s.: $P > 0.05$, sign test for differences from 50:50 distribution of predators over the two arms.

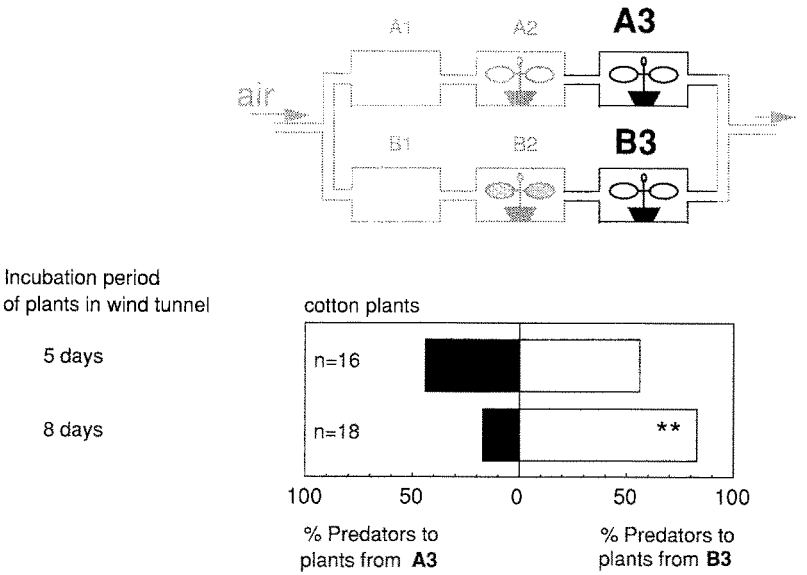


FIG. 6. Response of satiated *P. persimilis* females in a Y-tube olfactometer to plants from compartment A3 vs. plants from compartment B3. $^{**}P \leq 0.01$; n.s.: $P > 0.05$, sign test for differences from 50:50 distribution of predators over the two arms.

high. The adsorption hypothesis also is not supported in control experiments in which uninfested plants were exposed to synthetic (*E*)-β-ocimene: only trace amounts of this compound were found in the headspace of the exposed plants. Alternatively, plants receiving information about nearby spider-mite infestations start producing the two bodyguard-attracting allelochemicals themselves. This can be interpreted as a strategy of "overhearing" other plants' cries for help and subsequently sounding the alarm. More detailed investigations into this strategy of plant defense are currently under way. In all these investigations, adsorption of allelochemicals to wind-tunnel walls as an explanation of the data has been precluded by taking plants out of the wind tunnel for behavioral or chemical investigations.

Differences between Plant Cultivars

Plant cultivars are known to differ in production of entomophage-attracting synomones (Van Emden, 1986; Elzen et al., 1985, 1986). For instance, genetically similar cotton cultivars and lines differ in quantities of parasitoid-attracting terpenes produced. Glanded cultivars produced ca. 100 times more terpenes than glandless cultivars (Elzen et al., 1985). For spider-mite-infested bean plants (*Phaseolus vulgaris*) differences in attractiveness for predatory mites have been found for two different cultivars. In a Y-tube olfactometer clean bean leaves were offered vs. spider-mite-infested leaves. *Phytoseiulus persimilis* females were attracted by pole bean leaves infested with 400 spider mites, but not by bush bean leaves infested by 400 spiders mites for the same period of time (two days). Bush bean leaves infested by 1000 spider mites are attractive to predators (Figure 7). Whether this is the result of quantitative or qualitative differences in the emitted blend of volatiles needs to be investigated.

ROLE OF PLANTS IN OTHER MULTITROPHIC SYSTEMS

So far, we have given details on the role of plants in the tritrophic system of predatory mites, spider mites, and several plant species. However, evidence for active involvement of plants in multitrophic interactions is appearing for other systems as well. Data supporting the idea of "talking plants" have been put forward by Rhoades (1983, 1985a,b) and Baldwin and Schultz (1983), although Fowler and Lawton (1985) criticized their results on methodological grounds. However, data on this issue are accumulating and recent research efforts have shown phenomena that are similar to those described for the mite–plant system.

Using an olfactometer, Nadel and Van Alphen (1987) showed that cassava plants infested by cassava mealy bug attract the parasitoid *Epidinocarsis lopezi*: not only the infested leaves are attractive but also uninfested leaves of infested

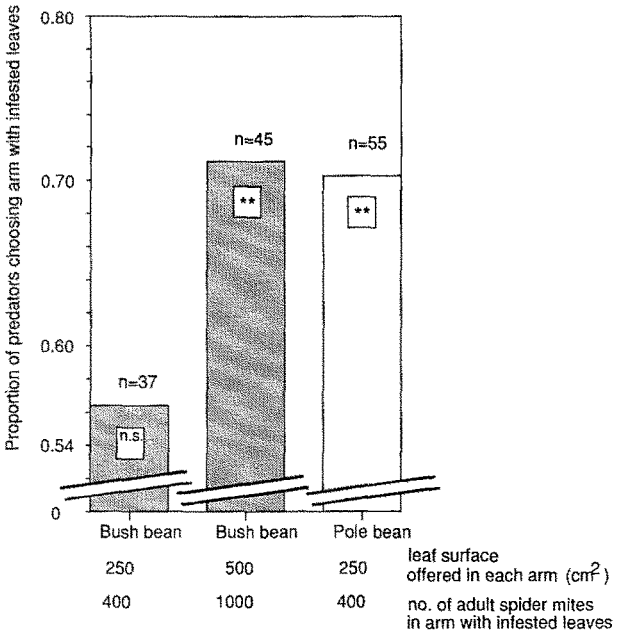


FIG. 7. Response of satiated *P. persimilis* females in Y-tube olfactometer experiments with different *Phaseolus vulgaris* cultivars infested by *T. urticae*. Infested leaves were offered vs. uninfested leaves of the same cultivar. *Phaseolus vulgaris* cultivars: (1) Bush bean 'dubbele zonder draad,' nr. 697 and (2) polen-bean 'Westlandse dubbele' nr. 671, Turkenburg Ltd., The Netherlands $^{**}P \leq 0.01$; n.s.: $P > 0.05$, sign test for differences from 50:50 distribution of predators over the two arms.

plants are more attractive than uninfested leaves of uninfested plants. Unfortunately, they did not try to avoid adsorption of volatiles emitted from the infested leaves. Thus, it remains unknown whether the uninfested leaves actually produce the allelochemical.

Turlings et al. (1990) reported behavioral and chemical data for a tritrophic system of the parasitoid *Cotesia marginiventris*, noctuid herbivores, and corn plants. It appears that phenomena that are quite similar to some of those reported here for the mite-plant system are found in their parasitoid–herbivore–plant system: after damage by the herbivore, plants emit chemicals of plant origin that attract parasitoids.

The most intriguing data on active involvement of plants in defense were published for a system of plants and their pathogens (Zeringue, 1987). It was shown that cotton plants that receive odors of conspecifics infested with a pathogen start production of phenols that hamper pathogen growth. This compares to the findings that plants that have been in the neighborhood of spider-mite-

infested plants attract more predatory mites than control plants that have not experienced spider-mite-infested plants.

PROSPECTS FOR APPLICATION

In the earlier section on application in pest control, we summarized the prospects for integrating the use of allelochemicals in pest management strategies. One may ask whether these prospects apply to the mite-plant system discussed subsequently and whether the results obtained in studying this system give rise to new avenues for pest control. Here, we will present our current thoughts.

1. Bringing the natural enemy in a specific search mode (Gross, 1981; Vet, 1988). The plant-related predator-attracting allelochemicals are involved in learning by predatory mites. Predators that have experienced *T. urticae*-infested Lima bean leaves prefer infested Lima bean leaves when given a choice between *T. urticae*-infested Lima bean and *T. urticae*-infested cucumber leaves. However, if these predators are given a seven-day experience on *T. urticae*-infested cucumber leaves they prefer infested cucumber leaves (Figure 1) (Dicke et al., 1990b). These recent data have not yet led to ideas for application.

2. Making artificial host–prey species acceptable in a mass rearing program (Vinson, 1986). Nonvolatile kairomones are involved in prey acceptance (Jackson and Ford, 1973), but no evidence for an effect of volatile allelochemicals on prey acceptance has been shown. Data on prey preference have shown that volatile kairomone preference is correlated with prey preference (Dicke et al., 1988), but no causal relationship has been reported. We do not expect applications in this context.

3. Using the response to the allelochemical as a criterion in selection of natural enemies for biological control. This valuable idea is being used to develop biological control of the cassava green mite in Africa (Janssen et al., 1990). Its contribution to a reduction in time needed for prerelease evaluation programs of predatory mites may be expected within five years. This application is not dependent on the detailed knowledge presented in this paper, however.

4. Using the response to the allelochemical as a criterion in quality control (Dicke et al., 1989c; Noldus, 1989). The response to volatile allelochemicals appears to be crucial in the ability of a predator to exterminate a prey population (Sabelis and Van der Meer, 1986), and thus incorporating this response in a quality control program may be worthwhile, the more so because data are available for loss of the response to volatile allelochemicals in small-scale laboratory rearings of predatory mites (Dicke et al., 1989c). This application may be manifest today, but so far quality control programs for natural enemies are scarce (Bigler, 1989).

5. Enhancing searching efficiency of entomophages (Lewis et al., 1975a,b, 1979; Gross, 1981). As shown above ("Allelochemicals affecting foraging behavior of natural enemies and application in pest control"), application of this idea in the *Heliothis–Trichogramma* system suffered from problems of distributing the allelochemical in the field (Gross, 1981). Compounds that inform beneficial arthropods about herbivore location should be applied according to the actual distribution of the herbivores and the behavioral response of the natural enemies, otherwise the beneficials are misled instead of supported. Our data for the mite-plant system show that plants are involved in production of volatile allelochemicals that attract predatory mites to and retain them in spider-mite patches. Because differences in attraction have been found for different bean cultivars infested by *T. urticae*, plant breeding may yield cultivars that emit higher concentrations of predator-attracting allelochemicals. Thus, enhancing predator efficiency by breeding plants for higher concentrations of allelochemicals (proposed for allelochemicals that are emitted without herbivore damage; Nordlund et al., 1981b; 1988) may bring new application possibilities. For these possibilities to become reality, it is necessary to convince plant breeders that plant resistance against herbivores has not only a direct component but also an indirect component, i.e., through the action of the third trophic level (Price et al., 1980). Neglecting the indirect component in a plant breeding program may lead to elimination of indirect defense and thus to selection of cultivars that have a reduced net resistance under field conditions, especially in those cases where natural enemies play a significant (but often unnoticed) role in reduction of herbivore populations (see Van Emden, 1986, for an example related to allelochemicals). Alternatively, plant breeders may aim at selecting a cultivar in which searching behavior of a natural enemy is not hampered, but rather improved. This has been done successfully for a morphological trait of cucumber plants in an attempt to have satisfactory control of whiteflies by the parasitoid *Encarsia formosa*. High hair densities on leaves of cucumber varieties used to date in horticulture impede host searching by the parasitoid, and, thus, biological control is not successful on these varieties (Hulspas-Jordaan and Van Lenteren, 1978; Van Lenteren et al., 1987; Li et al., 1987). Recently, new cucumber varieties with lower hair density have been selected in a cooperative effort of plant breeders and entomologists. Greenhouse experiments showed that parasitism of whiteflies by *Encarsia formosa* was significantly higher on a cucumber variety having a lower hair density than the variety used by growers (Van Lenteren and De Ponti, 1990). Such approaches to application may be feasible within 10 years.

We expect that taking indirect plant defense tactics more seriously will enable man to effectively cooperate with plants in the battle against herbivores. Allelochemicals will prove to be useful manipulators.

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SEMIOCHEMICALS AND LEARNING IN PARASITOIDS

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Abstract—There is increasing evidence that parasitoid responses to semiochemicals, important stimuli in the host location process, are influenced by experience. This paper focuses on the role of learning, in particular associative learning, in responses to odors. Emphasis is placed on associative learning during the adult stage, the influence of preadult experience is discussed briefly. New data on learning in the species *Leptopilina heterotoma* are given. It is demonstrated that females can learn to respond to a novel odor, which they subsequently use in microhabitat selection. Learning was shown to be associative whereby host products (contact kairomone) or an oviposition experience could function as the reinforcers (reward). The effect of learning seemed stronger when parasitoids were rewarded with an oviposition experience. The paper concludes with a discussion on the application of parasitoid learning in pest management.

Key Words—Learning, odor, semiochemicals, parasitoid, biological control, *Leptopilina heterotoma*, Hymenoptera, Eucoilidae.

INTRODUCTION

Being part of a tritrophic system of plants, phytophagous insects and their natural enemies, parasitoids have a great opportunity to use semiochemicals in their search for suitable hosts in which to lay their eggs. They can use information coming from either the food of the host, the host itself, from an interaction of the two, and from host-associated organisms. Knowing the impressive diversity of behavioral modes with which different parasitoid species search for their hosts, it is not surprising to find a corresponding interspecific diversity in

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the type and source of semiochemicals used. Synomones and kairomones of very different origins may induce behavioral responses such as flight, arrestment, antennation, ovipositor probing, or oviposition. Each species has its unique repertoire of stimuli and corresponding responses. Evidence of the role of semiochemicals in the foraging behavior of parasitoids has been reported in several recent reviews (for references see Noldus, 1989).

Detailed study of specific parasitoid–host systems and comparison between systems may enable us to infer the adaptive significance of specific stimulus–response patterns (see, e.g., Vet and van Alphen, 1985). However, although it is interesting to understand how and why response patterns differ, it is equally interesting to look for generalities in their mode of occurrence. One of these generalities is the recognition of a significant amount of variation in behavioral responses within each species. Behavioral repertoires are complex processes influenced by genes, the physiological state of the animal, the environment, experience, and interactions between these factors (Vet et al., 1990b; Lewis et al., 1990). The present paper focusses on the role of experience on foraging behavior in parasitoids, in particular, on their responses to volatile allelochemicals.

After a brief review of the present knowledge on preadult experience, we will focus on the influence of experience, especially associative learning, during the adult stage. New specific data on associative learning in the species *Leptopilina heterotoma* (Eucoilidae) will be given. Finally we will discuss prospects for application of parasitoid learning in pest management.

Preadult Experience. Parasitoids develop in and emerge from a host that has fed on a certain diet. There is some evidence that this specific environment can influence behavioral responses at the adult stage (e.g., Thorpe and Jones, 1937; Vinson et al., 1977; Smith and Cornell, 1978; Vet, 1983, 1985a; Luck and Uygun, 1986; Sheehan and Shelton, 1989). For example, the response of adult females of the eucoilid parasitoid *Leptopilina clavipes* (a parasitoid of *Drosophila*) to yeast odors was increased by rearing them as larvae in hosts on yeast (Vet, 1983). Similarly, the aphid parasitoid *Diaeretiella rapae* showed a higher response to the odor of potato when it was reared on aphids feeding on this host plant (Sheehan and Shelton, 1989). The larval parasitoid *Microplitis demolitor* reared from *Heliothis zea* larvae fed on cowpea plants responds strongly to the odor of infested cowpea plants, whereas females reared from hosts fed on artificial diet do not (Hérard et al., 1988). The polyphagous braconid *Aphaereta minuta* only distinguished between odors from host-infested and uninfested substrates when it was reared in that substrate (Vet, 1985a). The mechanism behind such culturing effects is generally unknown. Confounding effects of artificial selection may be involved (Papaj and Rausher, 1983), as they are often difficult to rule out.

It appears most likely that the adult parasitoid's response is modified prior

to or during eclosion through a chemical legacy from previous developmental stages (Vet, 1983, 1985a; Corbet, 1985). After all, the sensory system matures during the late pupal stage of the parasitoid. A clear distinction between preadult and adult effects of experience on adult behavior is difficult to make (Hérard et al., 1988). However, in terms of assessing the impact of learning on the foraging success of the parasitoid, such a distinction may not be all that important to make in the first place.

Adult Experience. In contrast to the ambiguity surrounding preadult effects of experience on adult behavior, it is quite clear that experience by the adult often does affect its subsequent behavior. Experience during the adult stage appears to have much more impact on subsequent behavioral responses than experience during development (Vinson et al., 1977; Sandlan, 1980; Vet, 1983; Drost et al, 1988; Sheehan and Shelton, 1989), a conclusion that also has been drawn for other insects such as *Drosophila* species (Jaenike, 1983). Adult learning, where learning is defined loosely as "any change in behavior with experience" (for a discussion of the definition of learning, see Papaj and Prokopy, 1989), may have an impact on every phase of parasitoid foraging from habitat location (e.g., Sheehan and Shelton, 1989; Papaj and Vet, 1990) to host acceptance (e.g. Cornell and Pimentel, 1978; Kaiser et al., 1989).

Associative learning (defined as the establishment through experience of an association between two stimuli or between a stimulus and a response) is an often suggested learning mechanism (e.g., Vinson et al., 1977; Lewis and Tumlinson, 1988; Turlings et al., 1989; Vet, 1988 and see below; Ding et al., 1989). However, a rigid control for nonassociative processes such as sensitization and habituation is not always incorporated, but this is often difficult to do (Papaj and Prokopy, 1989). Through associative learning, parasitoids may increase a preexisting but weak overt response to a stimulus (i.e., so called "alpha conditioning"; see Carew et al., 1984; Gould and Marler, 1984). In a natural context, alpha conditioning may be a common and very functional kind of associative learning in parasitoids. Response increases to stimuli through learning that is most likely associative have been demonstrated in several parasitoid species including braconids (Vinson et al., 1977; Vet and van Opzeeland, 1984; Drost et al., 1986, 1988; Turlings et al., 1989, 1990), eucoilids (Vet, 1983, 1988; Vet and van Opzeeland, 1985; Vet and Schoonman, 1988; Papaj and Vet, 1990), aphidiids (Sheehan and Shelton, 1989), tachinids (Monteith, 1963), ichneumonids (Arthur, 1966, 1971; Sandlan, 1980; Wardle and Borden, 1985), and trichogrammatids (Kaiser et al., 1989). Most of these species (except for parasitoids of Drosophilidae) are natural enemies of economically important insect pests.

Although parasitoids of *Drosophila* flies are of no great applied importance, the searching and parasitization behavior of some of these species is relatively well studied (van Alphen and Vet, 1986). Most parasitoid species are

microhabitat specialists, i.e., they attack hosts in either fermenting substrates (such as fruits), decaying plant materials or mushrooms. Others, such as *Leptopilina heterotoma*, are more generalists and have been reared from several *Drosophila* species in several microhabitats (Vet and van Alphen, 1985; Janssen et al., 1988). The searching behavior of these species is guided by several allelochemicals. Important ones are volatiles from the food of the hosts that are used for microhabitat location and selection (see references in van Alphen and Vet, 1986). After arrival in the microhabitat, nonvolatile host cues (contact kairomones) are used for indication of host presence and possibly host suitability (Dicke et al., 1985; Vet, 1985b; van Alphen and Vet, 1986).

For a number of *Drosophila* parasitoids, it has been shown that responses to microhabitat odors are strongly influenced by adult foraging experience through an alpha-conditioning process (Vet, 1983, 1985a, 1988; Vet and van Opzeeland, 1984, 1985; Papaj and Vet, 1990). *Leptopilina heterotoma* females, for example, showed a dramatic increase in initially weak responses to host substrate odors after having experienced them in association with oviposition in host larvae. Females experienced with apple-yeast substrate respond significantly stronger to the odor of an apple-yeast substrate than naive females or females experienced with another substrate both in olfactometers (e.g., Vet, 1988) and in mark–recapture experiments (e.g., Papaj and Vet, 1990). Although evidence that experience influences the searching behavior and searching efficiency of this species abounds (see, e.g., Papaj and Vet, 1990), several aspects have remained unclear: (1) The associative nature of odor learning in this species has not been demonstrated unambiguously. (2) The reinforcing stimulus involved is still unclear: Is an actual contact with the host necessary to induce the behavioral changes? (3) It is unknown if odor learning in this species is limited to natural substrates or if parasitoids can acquire responses to other, novel, odors if these have been shown to be reliable predictors of host presence. The following olfactometer experiments were designed to clarify these issues. Different groups of females were given different "foraging" experiences while being exposed to an additional odor. Afterwards their response to this odor was tested. The novel odor involved a "green leaf volatile" (*cis*-3-hexen-1-ol) that does not occur in fermenting substrates (Williams et al., 1981) but to which *L. heterotoma* is known to show an electroantennogram response (Vet et al., 1990a).

METHODS AND MATERIALS

Parasitoids. Female *L. heterotoma*, aged 3–7 days, were obtained from a laboratory culture established from wasps collected from *Drosophila phalerata* in *Phallus impudicus* mushrooms in 1985 and reared since on this host on commercially grown mushrooms. Adult wasps were stored at 12.5°C after emer-

gence until at least 1 hr before being experienced, after which they remained at 20°C. All actions were performed at 20°C.

Substrates. For most treatments the basic substrate consisted of a suspension of a living-dry form of yeast (Engedura, Gist-Brocades, 50 g/100 ml water). Two milliliters of this suspension was spread out on a 2% agar layer, condensed by evaporation, and stored for three days at 20°C. Host-infested substrates were obtained by adding a slice of agar containing 100–200 2-hr-old *D. melanogaster* eggs before storage. This way the substrate, when used to give the wasps an oviposition experience three days later, contained larvae suitable for parasitization. Substrates containing kairomone were obtained by spreading 0.34 ml of a kairomone solution (see below) over the yeast shortly prior to experiencing the wasps (0.34 ml of water was added to substrates of the other treatments in same experiment). In the final experiment, the host-infested substrate was not achieved by letting larvae grow up in the yeast, but by adding about 100 late first–early second instar larvae, plus kairomone, to the substrate just prior to use.

Kairomone Solution. In a condensed yeast suspension containing 1.25 g of yeast, spread out on an agar layer in a Petri dish (5 cm diameter), 640 late first–early second instar larvae of *D. melanogaster* were allowed to feed for 20 hr at 20°C. Hereafter, 3 ml of water was added, and the suspension was filtered over moist filter paper (54 mm diameter). The filtrate, containing the water-soluble kairomone of about 200 larvae/ml, was divided into small aliquots of 0.34 ml and deep frozen (−20°C) until further use. Dicke et al. (1985) showed that freezing does not damage the kairomone's activity.

Treatments (General Procedure). Ten female wasps were confined in a jar similar to the ones used for odor sources with the olfactometer (see Figure 1, jar A). While the animals were confined, air was drawn from jar B through jar A, providing a possibility to draw an additional odor over the substrate with which the wasps were confined. This additional odor involved a green leaf volatile (*cis*-3-hexen-1-ol). Air was drawn over a 1% solution of *cis*-3-hexen-1-ol in paraffin oil (Ulvasol) (see Figure 1, jar B). This odor is henceforth indicated as *Z*3−6:OH. As a control, odor of pure paraffin oil was used both in the treatments and in testing (control air). All treatments were given the day prior to testing.

1. Naives. Females were confined for 1 hr in a clean agar jar while being exposed to control air (= odor of pure paraffin oil).

2. *Z*3–6:OH. Females were confined for 1 hr in a clean agar jar while being exposed to *Z*3–6:OH odor.

3. Y. Females were confined for 1 hr in a jar containing uninfested yeast substrate while being exposed to control air.

4. Y + H. Females were confined for 1 hr in a jar containing host-infested yeast substrate while being exposed to control air.

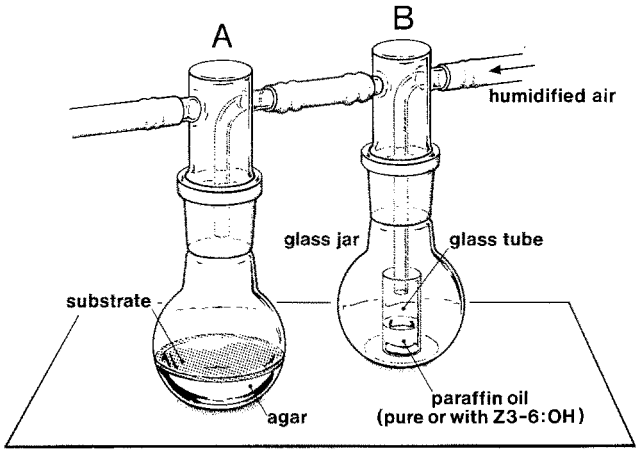


FIG. 1 Training set-up used in the learning experiments. Air is sucked through vial B containing 1 ml paraffin oil (pure or with a 1% *cis*-3-hexen-1-ol solution), after which it is drawn through jar A in which females are confined. The substrate in jar A differed for the different treatments.

5. Y + H + Z3–6:OH. Females were confined for 1 hr in a jar containing host-infested yeast substrate while being exposed to Z3–6:OH odor.

6. Y + Z3–6:OH. Females were confined for 1 hr in a jar containing uninfested yeast substrate while being exposed to Z3–6:OH odor.

In the following two treatments, the amount of kairomone present in each jar was standardized by adding kairomone or kairomone and hosts to the uninfested yeast substrate shortly before confining the wasps.

7. Y + K + Z3–6:OH. Females were confined for 1 hr in a jar containing uninfested yeast substrate with kairomone added, while being exposed to Z3–6:OH odor.

8. Y + K + Z3–6:OH + 3 ov. Individual females were confined in a jar containing yeast substrate with kairomone and host larvae added, while being exposed to Z3–6:OH odor. They were removed after having oviposited three times.

Olfactometer experiments. Bioassays were performed in a four-armed airflow olfactometer, as described by Vet et al. (1983) with some minor modifications. The Perspex exposure chamber was replaced by a bottom layer of aluminum (to which the central introduction tube is connected) covered by a glass lid that rested on a rubber O-ring. In this olfactometer, four distinct odor fields can be created, and individual animals can move freely through the exposure chamber exploring the different fields. For further details we refer to Vet et al. (1983). For each female we recorded odor field first chosen, time allo-

cation to the different fields during a period of 5 min, and odor source finally chosen (female walks out of test chamber towards one of the odor sources without returning within 1 min, in which case the remaining test time is added to this field). As these parameters are generally highly correlated, figures throughout the paper are restricted to data on time allocation, while data on first and final choices are merely mentioned in the text when they add extra information. During each day, the same odor sources were used for all treatments, and females from the different treatments were tested alternately.

In the first experiment, one field of the olfactometer consisted of the odor of Z3–6:OH, while the remaining three fields consisted of air drawn over pure paraffin oil (control air). In the other experiments, yeast substrate was used as an odor source in two adjacent arms: 5 ml of the previously described yeast suspension was spread out on agar in an olfactometer jar, condensed, and stored for three days at 20°C. To one of these yeast odor fields, we added the odor of Z3–6:OH by drawing the yeast odor over a 1% solution of Z3–6:OH. Odor from the other three arms was drawn over pure paraffin oil.

RESULTS

Experiment 1. The first experiment was designed to determine: (1) the behavioral response of naive females to the novel odor; (2) if exposure to the odor alone would change the female's subsequent behavioral response to it; and (3) if females would change their subsequent response to the novel odor after having encountered it during an oviposition experience (treatments 1, 2, and 6, respectively). One odor field of the olfactometer consisted of Z3–6:OH odor, while three fields were created with control air.

Figure 2 shows the mean percent time spent per odor field for the three different treatments. Naive females seem to be slightly repelled by the Z3–6:OH odor, shown by the significant difference between the time spent in the Z3–6:OH field and one of the air fields (multiple comparisons, based on Friedman rank sums, $P < 0.05$). This repellency of Z3–6:OH odor was more apparent in the percentage females that made a first choice for a particular field (data not given in figure). Only 3% of the females tested made a first choice for the Z3–6:OH odor field while 97% of the females chose for air (chi-square test, $P < 0.005$). The percentage of females that showed an anemotactic response by walking out of the test chamber towards an odor source (final choice) was very low for all treatments in this first experiment, hence these were not analyzed statistically.

Females previously exposed to Z3–6:OH did not respond differently to air or Z3–6:OH odor. Z3–6:OH-exposed females apparently were no longer repelled by the odor of Z3–6:OH, a change in response that could be ascribed

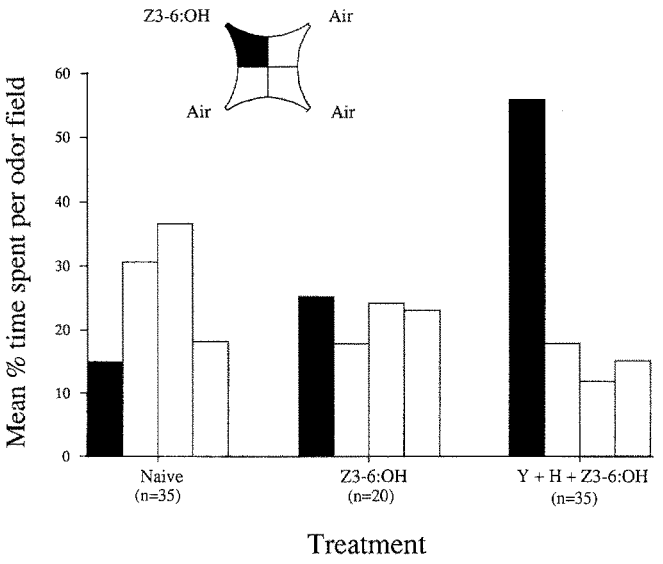


FIG. 2. Response (mean percentage time spent per odor field) of *L. heterotoma* females with different previous experiences (see Methods and Materials) to the odor of *cis*-3-hexen-1-ol (Z3-6:OH) produced in one arm of the olfactometer. The other three arms produced control air only. Y = yeast substrate; H = host larvae present in yeast substrate.

to a form of habituation. Comparison of the data for naive and Z3-6:OH-exposed females, however, did not reveal any statistical differences. (Time spent in the Z3-6:OH field: Mann-Whitney U test, $P < 0.05$; first choices for Z3-6:OH versus air fields, G test: $P > 0.05$). We concluded that females can habituate to the novel odor but that strong sensitization to the novel odor, i.e., females becoming attracted to it, was lacking.

Results for the third treatment, whereby females had been exposed to the novel odor while ovipositing, clearly show an increase in response to the novel odor. Significantly more time is spent in the Z3-6:OH field as compared to the air fields (Figure 2; Friedman two-way ANOVA by ranks, $P < 10^{-7}$). Furthermore, the number of first and final choices is highest for the Z3-6:OH field. Of 35 females, 22 made a first choice for the quarter of the olfactometer that offered the Z3-6:OH field, which is highly significant (chi-square test, $P < 0.001$). Only 10 of the 35 females tested made any final choice, six of which were for the Z3-6:OH odor.

Experiment 2. In the second experiment we combined the novel odor with the odor of a yeast substrate. In one half of the olfactometer, parasitoids could now choose between yeast odor with or without Z3-6:OH odor. Two arms

offered control air. We extended the number of treatments to test rigorously if associative learning was involved or whether the increase to the novel odor in the first experiment was due to a general increase in responsiveness to odors after an oviposition experience. Treatments involved naive females, females with exposure to an uninfested yeast substrate, females with oviposition experience in hosts in yeast, and finally females with oviposition experience in hosts in yeast while being exposed to Z3–6:OH odor (treatments 1, 3, 4, 5, respectively).

Results of the time allocation to the four different odor fields for the different treatments are presented in Figure 3. Naive females and females exposed to an uninfested yeast substrate distributed their time equally over the four fields. These results also indicate that there was no sensitization to the substrate odor by mere exposure to the substrate, a control that has often been lacking in parasitoid learning studies.

Females with an oviposition experience in yeast (without ever having been exposed to Z3–6:OH odor) spent significantly more time in the field with yeast odor than in the yeast odor field with Z3–6:OH added (Wilcoxon matched-pairs signed-ranks test, $P < 0.05$). It seems that females can distinguish between the two yeast odor fields and prefer the odor of "clean" yeast.

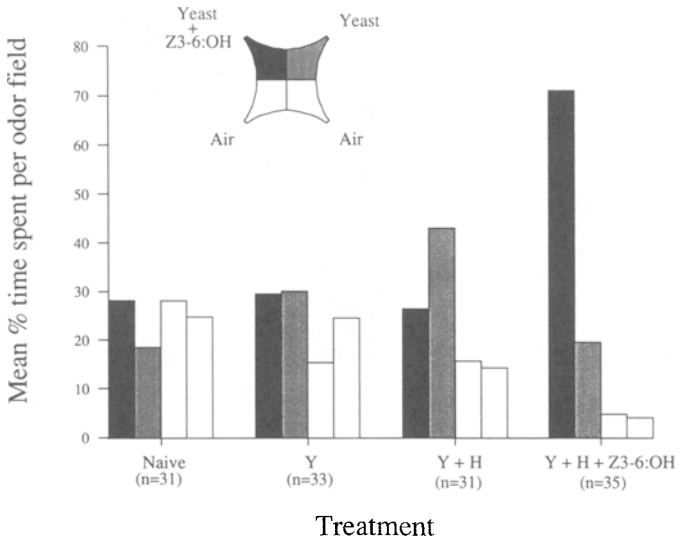


FIG. 3. Response (mean percentage time spent per odor field) of *L. heterotoma* females with different previous experiences (see Methods and Materials) to the odor of yeast with and without the additional *cis*-3-hexen-1-ol (Z3–6:OH) odor. The two remaining arms of the olfactometer produced control air only. Y = yeast substrate; H = host larvae present in yeast substrate.

Females of the final treatment, which oviposited while being exposed to Z3–6:OH odor, significantly preferred the odor of yeast with Z3–6:OH added. These results clearly show that females are capable of associating the experience of oviposition with the presence of a novel odor and that they can use this information in subsequent microhabitat selection.

Experiment 3. The third experiment was designed to investigate the possible role of an encounter of kairomone as a reinforcer (reward), and to compare this to the role of oviposition experience as a reinforcer (treatments 7 and 8). We standardized and equalized the amount of kairomone present in the substrate by adding kairomone only or kairomone plus suitable host larvae immediately prior to confining the wasps. Females in treatment 8 were allowed—and individually checked—to oviposit three times, after which they were removed. Furthermore, we added a rigorous control for sensitization (treatment 6). The olfactometer setup was identical to the previous experiment. Results are shown in Figure 4. A kairomone contact was sufficient to learn the novel odor (time allocation, $P = 0.05$; first choice, $P < 0.05$; final choice, $P < 0.01$). The

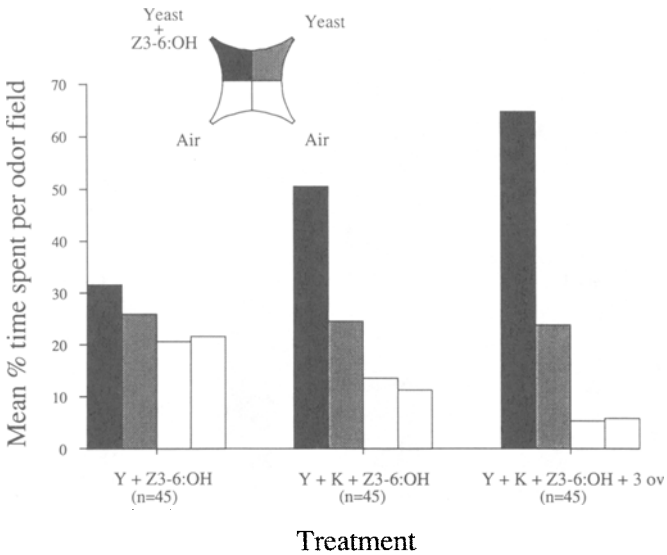


FIG. 4. Sensitization control and comparison of a kairomone and an oviposition reward. Response of *L. heterotoma* to the odor of yeast with an without the additional *cis*-3-hexen-1-ol (Z3–6:OH) odor. The two remaining arms of the olfactometer produced control air only. Females had been exposed to Z3–6:OH odor, while either being confined for 1 hr in a jar containing uninfested yeast (Y) substrate without or with kairomone (K) added, or they had been allowed to oviposit three times (3 ov) in hosts in yeast substrate to which kairomone and hosts were added.

learning effect was more pronounced in the treatment where females oviposited (time allocation, $P < 0.05$; first choice, $P < 0.01$; final choice, $P < 0.001$). Comparison of time allocation between the two treatments revealed, however, no significant difference (Wilcoxon matched-pairs signed-ranks test, $P = 0.40$ based on time spent in the yeast plus Z3 – 6:OH odor field relative to the time spent in both yeast odor fields). Being aware that this nonparametric test is a relatively weak test of difference in distributions, we analyzed the number of choices females made for the two different yeast odor fields, using a parametric test. Percentages of choices for a particular odor field were calculated per two test days, after which they were transformed with an arcsin transformation. Under the assumption that these transformed data were normally distributed, differences between treatments were tested with a t test. However, this test did not provide significant statistical support either for the contention that oviposition experience induced a more pronounced learning effect ($P = 0.19$ and $P = 0.07$ for the first and final choices, respectively). The only statistical difference between the two treatments was in the time spent in both yeast fields compared to the air fields. Females with an oviposition experience were significantly more prone to spend time in the yeast odor fields compared to females with a kairomone experience only (Wilcoxon matched-pairs signed-ranks test, $P < 0.05$).

Finally, females exposed to Z3–6:OH odor, while confined with uninfested yeast substrate, did not distinguish between the yeast odor fields. Hence, once more we concluded that sensitization was not involved.

DISCUSSION

Our results clearly show that *L. heterotoma* can acquire responses to novel odors through a process of associative learning. Nonassociative processes such as habituation and sensitization were shown to be of minor and no importance, respectively. Apparently, learning in microhabitat location needs not be restricted to odors emanating from the microhabitat itself, odors with which parasitoids are naturally acquainted. Under the constraint that stimuli need to be sensorily perceived, parasitoids can perhaps incorporate any stimulus that has been shown to be a reliable predictor of host presence and suitability into their behavioral repertoire. This creates a tremendous possibility for individual parasitoids to enhance their foraging efficiency, and it can generate considerable intraspecific variation in searching behavior.

Furthermore, we can conclude that in this learning process only stimuli derived from the second trophic level (the host itself) act as the reinforcers, for the novel odor is not learned by linking it to a "successfully discovered" microhabitat (the yeast substrate) but only by linking it to hosts or host products. For

naive parasitoids that initiate foraging, this is the most reliable way to extend the set of stimuli to which they will further respond. Vet et al. (1990b) speculate that perhaps later during foraging other kinds of stimuli can achieve a function as reinforcer through a process of second-order conditioning, whereby a stimulus that is learned becomes itself a reinforcer. This, however, has not yet been tested for parasitoids.

Until recently, the reinforcer was generally assumed to be a stimulus involved in successful oviposition, perhaps an egg-releasing stimulus. However, as our results show, an actual contact with the host is not necessary to learn the novel odor. Similar findings have been reported for three parasitoid species attacking lepidopterous larvae (Lewis and Tumlinson, 1988; Ding et al., 1989; Turlings et al., 1989), and it may be a common phenomenon for species where the presence of host products strongly predicts the presence of suitable hosts. For parasitoids of *Drosophila*, contact kairomones are an important source of information on host quantity (van Alphen and Galis, 1983) and quality (Vet, 1985b; van Alphen and Vet, 1986). Naive female *L. heterotoma* show an innate response to contact kairomone in that they probe slightly but consistently faster with their ovipositor in patches that contain kairomone than in patches without kairomone (each female tested on both patches: Wilcoxon-matched pairs signed-ranks test, $P < 0.03$, $n = 54$; Groenewold, unpublished). However, interestingly enough, response to kairomone expressed in the time spent searching on a patch is absent in naive females and is only evident after an oviposition experience (Vet, 1985b). Similar findings are reported for *Asobara tabida,* another parasitoid of *Drosophila* (Vet, 1985b). These findings led to the premature conclusion that kairomones are only recognized after an oviposition experience; hence that kairomones need to be learned. The present results, whereby a kairomone is used by naive females as a reinforcer in a learning process, are additional evidence that these host-derived products are innately recognized. However, it may be an adaptive strategy for naive parasitoids to increase their patch time in response to a kairomone only after it has been encountered in combination with actual oviposition. Oviposition does, after all, remain the ultimate proof of host presence. Furthermore, responses to kairomones can additionally be modified by learning: for *Asobara tabida* there is indeed indication that they can learn to distinguish between kairomone of *D. melanogaster* and kairomone of their preferred host *D. subobscura* (van Alphen, unpublished; van Alphen and Vet, 1986).

Oviposition, being the only genuine proof of host presence, and to some extent of host suitability, can be expected to give a stronger reinforcement effect as compared to kairomone as a reward. This was indeed strongly suggested by our data (Figure 4), where learning seems more pronounced after an oviposition experience. Similarly, for the larval parasitoid *Microplitis croceipes*, it has been shown that reinforcement with oviposition gives a stronger learning effect than

experience with host-derived frass only, in that parasitoids remember the learned information longer after an oviposition experience (Martin and Lewis, in preparation). For a more detailed discussion on how different reinforcements can influence responses to stimuli see Vet et al. (1990b).

APPLICATION OF LEARNING IN PEST CONTROL

During the last five years, there has been an increasing recognition of learning as an important source of behavioral plasticity in parasitoids, and we expect that aspects of learning will become a major consideration in studies on parasitoid – host and parasitoid – environment relationships. In practically all cases where learning in parasitoid foraging was investigated, it was successfully demonstrated (but see Vet et al., 1990b). However, until now no serious attempt to apply learning to improve natural enemy effectiveness has been made. Although evidence that parasitoids can learn abounds, there is still little insight in how and if experience affects foraging efficiency. Wardle and Borden (1986) showed that prior experience of wasps (*Exeristes roborator*) with an artificial rearing system (hosts in plastic egg cups) significantly reduced responses to the habitat of a natural host, and the authors warn of adverse effects of learning in artificial insectary systems. The results of the present paper, showing that novel, perhaps more "unexpected," odors can be learned and therefore be incorporated in the foraging process, are yet another warning against aberrant learning that could interfere with learning of target stimuli.

To our knowledge there are only two studies conducted under field conditions that show learning can improve a parasitoid's foraging success (Papaj and Vet, 1990; Lewis and Martin 1990). It is promising, however, that, at least for *L. heterotoma*, these field experiments affirm previous laboratory findings on odor learning. For example, the low responsiveness of naive females in the olfactometer agreed with the low number of naive parasitoids that engaged in foraging for the target stimuli (microhabitat baits, i.e., patches of *Drosophila* substrate). By allowing females to associate the target stimuli with the presence of hosts, through an oviposition experience, females could be significantly "focused" to the target stimuli (Papaj and Vet, this issue). In several other studies it was shown that not only the mean response to a stimulus can be increased through learning but that learning simultaneously decreases the variability of the behavioral response and thus increases the predictability of its occurrence (Vet et al., 1990b). If reinforcement with host-derived products (without having to oviposit) proves to be effective in inducing such behavioral changes, this would increase the feasibility of implementing such practices. For this, it is promising that a reinforcing effect of kairomone in learning has been reported for three parasitoid species attacking economically important insect pest species (*Macrocentrus grandii*, Ding et al., 1989; *Microplitis croceipes*,

Lewis and Tumlinson, 1988; *Cotesia marginiventris*, Turlings et al., 1989). This phenomenon is most likely the explanation for the results of some studies in the 1970s and early 1980s with *M. croceipes* and *C. marginiventris* showing, under laboratory and semifield conditions, that prerelease stimulation with kairomones could in fact significantly increase parasitization rates and reduce the tendency for released parasitoids to disperse (Gross et al., 1975; Gross, 1981; Loke and Ashley, 1984).

With present knowledge we can only speculate on further possibilities of using learning to improve the effectiveness of natural enemies in pest management, for example:

1. Aberrant learning during culturing should be avoided to prevent interference with learning of target stimuli.

2. The potential to respond to target stimuli should be improved through learning prior to release.

3. Maximal responses to target stimuli should be maintained by providing the right reinforcements during the foraging process.

With possibilities 1 and 2, we mainly aim to increase retention of the parasitoid population after release by increasing the focus of all individuals to the right stimuli. Post release migration of parasitoids away from the target area can be considerable (Gross, 1981; Ridgway et al., 1981; Keller et al., 1985; van Lenteren, personal communication). Reducing this could lead to an overall higher efficiency of the parasitoid population introduced (Gross, 1981; Lewis and Martin, this issue; Papaj and Vet, this issue). Knowing that parasitoids are apparently capable of learning novel odors can expand possibilities for application. Perhaps parasitoids can be taught to respond to specific and standardized odor sources most suitable to be used as odor flags in crops to initiate search and reduce dispersal of the parasitoids. However, even if such prerelease experience may increase the effective number of the parasitoid population used, it is still unknown if such initial experience could continue to enhance host encounter rate in the individual. Effects of habituation and negative reinforcements may counteract initial positive effects.

There is, as yet, no empirical support for possibility 3. The application of semiochemicals to crops has been attempted but results are not very consistent (Lewis et al., 1990). Knowledge of how repeated reinforcements influence foraging behavior is lacking and, again, more information is needed on the effect of habituation and negative reinforcement, especially in field studies.

The feasibility of using learning to enhance parasitoid effectiveness in pest management still has to be explored. At present we may only wonder to what extent incorrect "programming" of natural enemies was responsible for the lack of effectiveness in several of the inoculative or augmentative release programs in biological control.

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ODOR LEARNING AND FORAGING SUCCESS IN THE PARASITOID, *Leptopilina heterotoma*

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Abstract—A brief 2-hr experience with host *Drosophila* larvae in artificial apple-yeast or mushroom microhabitats had three effects on the foraging behavior of female *Leptopilina heterotoma* (Hymenoptera: Eucoilidae) parasitoids under field conditions. First, experienced females released at the center of circular arrays of apple-yeast and mushroom baits were more likely to find a microhabitat over the course of a daily census than naive ones. Second, for those females that found a microhabitat, experienced ones found it faster than naive ones (i.e., experience reduced travel times). Third, females experienced with a particular microhabitat were more likely to find that microhabitat than an alternative one. Learned preferences were retained for at least one day and possibly as many as seven. Results generally did not depend on the host species (*D. melanogaster* or *D. simulans*) with which females were given experience. Females tended to arrive at baits upwind of the point of release, suggesting that odor is involved in finding host microhabitats and, in particular, in learning to find them more effectively. The implications of these results for the application of semiochemicals in biological control are discussed briefly.

Key Words—Learning, odor, semiochemical, parasitoid, *Leptopilina heterotoma*, Hymenoptera, Eucoilidae, *Drosophila*, Diptera, Drosophilidae, biological control.

INTRODUCTION

Learning is often thought to be a mechanism by which an organism copes with the capricious nature of its environment (Papaj and Prokopy, 1988). Stephens

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(1987, 1990) noted, however, that learning is beneficial only so long as the environment is not too capricious. In his words, "Today must tell you something about tomorrow for learning to be worthwhile." Stephens (1990) invoked a haploid genetic model to argue that learning is most beneficial when the environment is unpredictable between generations but predictable within generations. In this scenario, learning permits successive generations to track long-term temporal changes in an environment but enables an individual within a given generation to detect short-term temporal regularities in the same environment.

Under such conditions, an individual's fitness ought to increase as it gains experience and adopts an appropriate behavior. With respect to the acquisition of essential resources such as food or breeding sites, for example, experienced individuals should forage more effectively than inexperienced (i.e., naive) ones so long as the environment remains relatively unchanged. For organisms that use chemical cues to orient to food or breeding sites, experience with predictable resources should improve their ability to use chemical stimuli to locate and handle those resources. As simple as this notion is, it has rarely been evaluated under conditions even remotely approximating natural ones.

With respect to biological control agents such as entomophagous parasitoids, this issue takes on practical as well as theoretical significance (see also Vet and Groenewold, 1990). Parasitoids can be useful biological control agents only to the extent that they effectively find hosts and so it is important to know whether or not experienced individuals forage more effectively than naive ones in the predictable environments represented by crop monocultures. In this regard, information about learning under field conditions would be enormously valuable. Here we examined how oviposition experience influences the effectiveness with which the parasitoid, *Leptopilina heterotoma*, forages for host *Drosophila* microhabitats under field conditions.

L. heterotoma (Hymenoptera: Eucoilidae) is a generalist endoparasitoid attacking a variety of *Drosophila* species inhabiting a variety of substrates, including rotting mushrooms, decaying vegetation, fermenting fruit, and sap fluxes. Under laboratory conditions, female *L. heterotoma* learn to orient to the odor of the microhabitat infested by their larval *Drosophila* host (Vet and van Opzeeland, 1985; Vet, 1988, and references within). Analysis of movement on a servo-controlled locomotion compensator (Vet and Papaj, in preparation) indicated that oviposition experience altered upwind responses to odor in a number of ways. Females walked faster and straighter, made narrower turns, and spent more time moving unwind in a plume of odor from a particular microhabitat if they had oviposition experience in that microhabitat than if they had experience in another microhabitat or no experience with larvae-infested microhabitats at all.

These results suggested two ways in which odor learning might improve

foraging success by parasitoids. First, experienced females should be more likely to find a microhabitat over the course of an assay than naive ones. Second, for those females that found a microhabitat, experienced ones should find it faster than naive ones. In addition, females experienced with a particular microhabitat should be more likely to find that microhabitat than an alternative one. These predictions are embodied in a central question addressed by our field experiments: if a given host microhabitat remains available in the environment, does a rewarding experience in that microhabitat improve the success with which a female *L. heterotoma* subsequently finds microhabitats of like type?

METHODS AND MATERIALS

Experience Protocol. Female wasps were obtained from a culture founded with wasps emerging from *Drosophila phalerata* in *Phallus impudicus* mushrooms in the De Horsten nature preserve in Wassenaar in 1987 and reared in the laboratory on *D. phalerata* in commercially grown mushrooms. Reproductively mature females between 1 and 2 weeks old were anesthetized with CO_2. A dot of paint (Decorfin, Talens Co., Apeldoorn, The Netherlands) ca. 0.5 mm in diameter was applied to the pronotum. The amount of paint used was enough to permit identification of the color with the unaided eye, yet not enough to hamper females in their movements. Equal numbers of females were marked with three different colors denoting the treatment to which females were subsequently assigned; different sets of colors were used in successive releases. The mark appears to affect neither survival nor behavior and mark loss is negligible over the course of a day (Driessen et al., in preparation).

Marked females were given one of three kinds of experience: (1) larvae-infested, fermenting apple-yeast substrate; (2) larvae-infested, decaying mushroom substrate; or (3) uninfested and plain agar (i.e., naive). Fermenting apple-yeast substrate was prepared by grinding up Golden Delicious apples in a Waring blender and adding a suspension of live bakers' yeast to the resulting mash in a volume–volume ratio of ca. 7 parts apple to 1 part yeast. This material was stored at 4°C and used within 48 hr of preparation. Decaying mushroom substrate was prepared by incubating commercial mushrooms on moist vermiculite for 3 days after the onset of sporulation. The decaying mushrooms were then ground up in a Waring blender and stored at 4°C until needed.

Apple-yeast or mushroom material was distributed on the bottom of an agar vial and infested with at least several dozen *D. melanogaster* or *D. simulans* larvae for 30 min prior to presentation to females. The 30-min waiting period was designed to permit larvae to produce a kairomone known to elicit host-searching behavior. Several hours after recovery from anesthetization, all females except naive ones were permitted to parasitize larvae freely. Naive

females were placed in an agar vial free of substrate and larvae. After 2 hr, all females were transferred to agar vials supplied with honey and stored at 12°C overnight. On the morning that females were released in the field, vials were brought gradually to room temperature and any remaining honey was removed.

Field Releases. Field releases were carried out in a forest on the outskirts of Wageningen in the Netherlands. The forest canopy was comprised of mixed beech and fir. The understory was sparse with scattered ferns and occasional bunches of grass. Host substrates such as rotting fruit or decaying mushrooms were absent in the immediate vicinity of our releases. Wasps were released at the center of a circular array of uninfested apple-yeast and mushroom baits (Figure 1). Baits consisted of circular patties 5.7 cm in diameter and 7 mm high and were distributed in pairs. Eight pairs each were arranged radially at distances of 3 and 6 m from the release point. Four pairs each were placed at distances of 12 and 18 m as shown in Figure 1. Members of a pair were placed ca. 50 cm apart. The left–right position of apple-yeast and mushroom baits within a pair was alternated systematically over the array and changed daily.

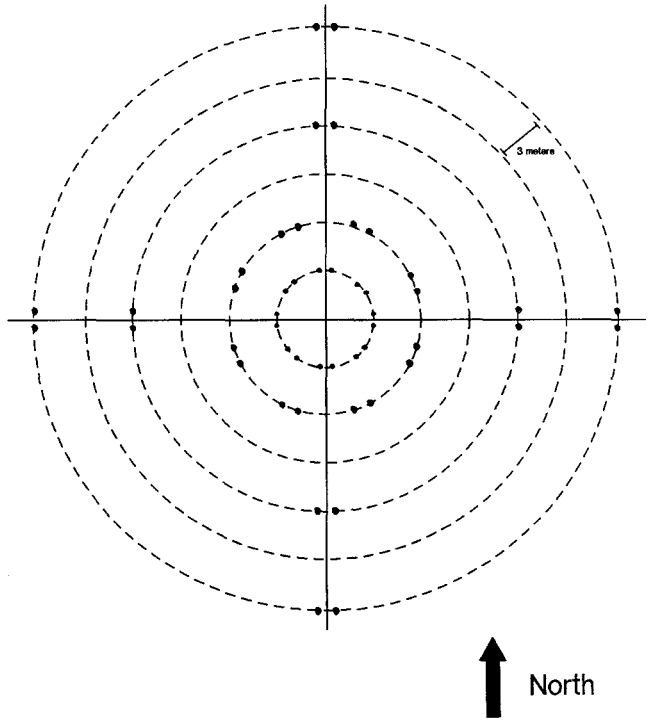


FIG. 1. Design of field array. Bullets indicate position of bait. Each pair included one apple-yeast bait and one mushroom bait. Female parasitoids were released at the center.

In the morning, wasps in different treatments were released simultaneously from vials placed at the center of the array. A check indicated that experience did not affect the time at which wasps left the vials. Fifteen minutes later, we censused each of the baits and collected by aspiration any wasps discovered on a bait. Censuses were generally repeated every 15 min thereafter until sundown. Occasionally, when initial captures were extremely low, censuses were made at 30-min intervals. We recorded the time and color code of each wasp as well as the location and type of bait on which it was found. We also recorded wind direction periodically with a hand-held compass and portable wind vane located within the array and wind velocity with a portable anemometer.

RESULTS

Upwind Movement by Foraging Parasitoids. By happenstance, winds were very strong and steady during the first three field releases and very light and variable during the last four. Figure 2 shows the distribution of wind directions

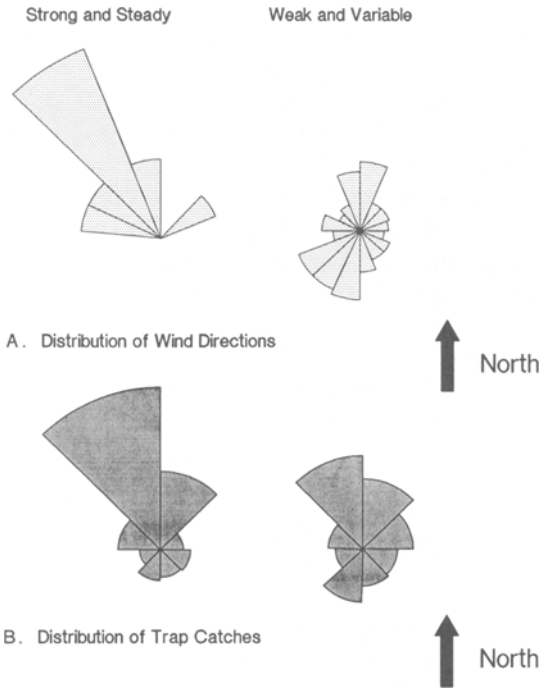


FIG. 2. Distribution of wind directions and trap catches for days on which wind was strong and steady and days on which wind was weak and variable. *N* for trap catches was 292 in strong and steady winds and 396 in weak and variable winds.

pooled over each time period and the corresponding distribution of captures at each bait station. When winds were strong and steady out of the northwest, female wasps exhibited a clear tendency to move in the upwind direction. When winds were light and shifting this tendency was much less pronounced, an observation confirmed by circular statistics. Rayleigh's r statistic for the distribution of trap catches was large and significantly different from 0 when wind was strong and steady ($r = 0.928$, $N = 26$; Rayleigh's test, $P < 0.05$) but not when wind was weak and variable ($r = 0.193$, $N = 80$; Rayleigh's test, $P > 0.5$). Taken together, these patterns suggest that females were moving upwind as we would expect if they were orienting to the odor of their host microhabitats.

Effect of Experience on Foraging Success. The percentage of females recovered at baits on the day they were released (i.e., percent capture) is shown in Table 1. Two patterns are readily apparent. First, proportionately fewer females in each treatment were recovered when winds were light and variable than when winds were strong and steady. This trend was highly significant (three-way G test, wind conditions × percent capture interaction, $G = 25.42$, $df = 1$, $P < 0.0001$; see also Table 1). Second, proportionately fewer naive females were recovered than experienced females of either type. This trend also was highly significant (three-way G test, experience type × percent capture interaction, $G = 59.24$, $df = 2$, $P < 0.0001$; see also Table 1). Finally, the effects of wind conditions and type of experience on percent capture were independent of one another (three-way G test, wind conditions × experience type × percent capture interaction, $G = 0.37$, $df = 2$, ns).

Experienced females were generally more likely to be found at baits than naive females released at the same time (Table 2). For females experienced with *D. melanogaster*, percent capture declined as the time between experience

TABLE 1. FEMALES CAPTURED AT BAITS ON DAY OF RELEASE WITH RESPECT TO WIND CONDITIONS AND TYPE OF EXPERIENCE[a]

	Capture when wind was			
	Strong and steady		Light and variable	
Type of experience	Released (*N*)	Captured (%)	Released (*N*)	Captured (%)
Apple-yeast	300	29.3 a1	660	19.6 b1
Mushroom	300	24.7 a1	660	14.4 b2
Naive	350	12.9 a2	550	7.6 b3

[a] Letters within a row (column) followed by the same letter (number) were not significantly different according to a pairwise G test at the 0.01 level.

TABLE 2. NUMBER RELEASED AND PERCENT CAPTURE ACCORDING TO TYPE OF EXPERIENCE AND NUMBER OF DAYS BETWEEN EXPERIENCE IN LABORATORY (IF ANY) AND RELEASE IN FIELD[a]

		Experience on			
		D. melanogaster		*D. simulans*	
Type of experience	Days since experience	Released (*N*)	Captured (%)	Released (*N*)	Captured (%)
Apple-yeast	1	185	40.5	160	30
	2	200	15.5	120	37.5
	4	100	2.0	140	11.4
Mushroom	1	185	31.4	160	16.9
	2	200	13.5	120	23.3
	4	100	7.0	140	13.6
Naive	1	335	11.3	160	5.6
	2	280	8.2	120	15.8
	4	40	12.5	140	2.1

[a] Data for females experienced with *Drosophila melanogaster* and *D. simulans*.

and release increased. By day 4, experienced females were captured at a frequency that was no different than that of naive females (Table 2). These results suggest that females given an opportunity to oviposit learned something that increased their tendency to find a microhabitat, but eventually forgot what they had learned. That this pattern was a consequence of forgetting and not due to aging is implied by the lack of a corresponding pattern among naive females.

Unlike females experienced with *D. melanogaster*, percent capture of females experienced with *D. simulans* did not decline steadily with time (Table 2). For example, percent capture of females experienced with *D. simulans* and released on day 1 was less than that of females released on day 2. Nevertheless, percent capture on day 4 was substantially lower than that on either days 1 or 2, a result consistent with that for females experienced with *D. melanogaster*. Moreover, a lower percentage of naive females were captured on each of the first two days than experienced females, a result consistent with that for females experienced with *D. melanogaster*. Overall, these results suggest that, like females experienced with *D. melanogaster*, females given an opportunity to oviposit into *D. simulans* learned something that increased their tendency to find a microhabitat, but eventually forgot what they had learned.

We performed a log-linear contingency analysis on these results. The patterns described above would lead us to expect a four-way interaction among

experience level, host species with which females were experienced, number of days between experience and release, and tendency to be captured. In fact, the experience type × host species × day of release × percent capture effect in the four-factor log-linear model was significant ($\chi^2 = 6.07$, $df = 2$, $P < 0.05$).

Effect of Experience on Travel Time. Of those females that found a bait over the duration of a daily census, experienced females found it faster than naive ones. For each day, we computed the overall median travel time to a particular bait for females in all treatments. We then tallied the number of experienced and naive females above or below that median. Finally, we summed these tallies across days. Results (Table 3) indicate that naive individuals tended to take longer than the overall median travel time to arrive at a bait. By contrast, experienced females tended to take less than the overall median time to reach a bait associated with successful oviposition. This difference is very significant (G test, $P < 0.003$). The difference between experienced and naive individuals persists even if we include the travel time of experienced females that arrived at the "wrong" bait, i.e., a bait different from that with they were experienced. Overall, ca. 54% of experienced females ($N = 259$) arrived at baits ahead of the overall median travel time, compared to only ca. 37% of naive females ($N = 82$). This difference is highly significant (G test, $P < 0.005$).

Our estimate of the effect of experience on travel time is likely to be a conservative one because we could only measure travel time for some individuals, specifically those individuals that arrived at a bait within our census period. Many females, however, never arrived at a bait; presumably some of those females might have found a bait had we censused over a longer period. Since many more naive than experienced females failed to be trapped at a bait within our census period, it is reasonable to suppose that naive females have even longer travel times relative to experienced ones than our crude analysis indicates.

TABLE 3. DISTRIBUTION OF TRAVEL TIMES TO APPLE-YEAST OR MUSHROOM BAITS BY EXPERIENCED AND NAIVE FEMALE PARASITOIDS[a]

Bait	Position about median	Experienced with bait	Naive
Apple-Yeast	Above	72	21
	Below	73	15
Mushroom	Above	52	31
	Below	62	15

[a] See text for additional explanation and analysis.

Effect of Experience on Microhabitat Choice. Apple-yeast-experienced females were recovered primarily at apple-yeast baits; mushroom-experienced females, by contrast, were recovered primarily at mushroom baits (Table 4). The frequency of captures at apple-yeast or mushroom baits was virtually identical whether individuals were released one or two days after experience (Table 4), suggesting that females did not forget over that time. Recovery of females released on day 4 was insufficient to draw any conclusions. Finally, learning did not appear to depend significantly on the host species with which females were experienced. These inferences were essentially confirmed by a log-linear contingency analysis including four factors—host species, type of experience, number of days between experience and release, and bait choice—which failed to identify any significant three-way or four-way effects.

The effect of experience on capture frequency at particular baits does not necessarily mean that females were orienting preferentially to the microhabitat associated with successful oviposition. The same result would be obtained if females foraged more persistently in those microhabitats. Mushroom-experienced females, for example, might be found more often at mushroom baits than at apple-yeast baits simply because they more often stay long enough to be

TABLE 4. NUMBER AND PERCENT OF INDIVIDUALS CAUGHT AT APPLE-YEAST BAIT ACCORDING TO EXPERIENCE AND NUMBER OF DAYS BETWEEN EXPERIENCE IN LABORATORY (IF ANY) AND RELEASE IN FIELD[a]

Days since experience	Type of experience	Experience on *D. melaogaster*		Experience on *D. simulans*	
		N	% at AY	*N*	% at AY
1	AY	75	77.3 a	48	87.5 a
	M	58	19.0 b	27	17.9 b
	NV	38	39.5 b	9	33.3 a
2	AY	31	77.4 a	45	93.3 a
	M	27	22.2 b	28	17.9 b
	NV	23	8.2 b	19	47.4 a
4	AY	2	19.5	16	43.8
	M	7	8.2	19	21.1
	NV	5	100.0	3	33.3

[a] Values for percent catches on apple-yeast close to 100 indicate a strong preference for apple-yeast; values for percent catches on apple-yeast close to 0 indicate a strong preference for mushroom. Data for females experienced with *Drosophila simulans* and *D. melanogaster*. Values within a column followed by the same letter are not significantly different at alpha level of 0.01 according to a pairwise *G* test (with Yates' correction where applicable). Data for day 4 were not analyzed due to insufficient sample sizes. AY = apple-yeast. M = mushroom. NV = naive.

censused. In fact, experience affects patch residence time in exactly this way under laboratory (Vet, 1988; Vet and Schoonman, 1988) and greenhouse conditions (Papaj and Vet, unpublished observations).

To examine whether census data could be explained on the basis of differences in patch time, we used laboratory data for *L. heterotoma* on apple-yeast and mushroom patches to estimate the distribution of captures. In our calculations, we assumed that (1) females were equally likely to arrive at apple-yeast and mushroom baits regardless of experience, and (2) oviposition experience on a particular substrate extended patch time in the field exactly as it did in the laboratory. Based on a total number of captures observed for females experienced with *D. melanogaster* and released day 1, results (Table 5) indicate that an effect of experience on patch time does not account adequately for the observed distribution of captures (Table 4). For both apple-yeast-experienced and mushroom-experienced females, the tendency to be captured at the microhabitat associated with previous oviposition was much more extreme than predicted by patch time alone. By contrast, the observed distribution of captures of naive females was not significantly different from that predicted by patch times.

In short, the first assumption underlying our estimate—that females are equally likely to arrive at apple-yeast and mushroom baits regardless of experience—is probably invalid. Rather, oviposition experience in a microhabitat appears to generate a tendency to orient preferentially to that microhabitat in the field. This interpretation is supported by results of greenhouse assays where individual females were followed from release to arrival at a patch (Papaj and Vet, unpublished data).

Females Captured on Subsequent Days. Although most females were cap-

TABLE 5. ESTIMATED DISTRIBUTION OF CAPTURES BASED ON PATCH TIME

Type of experience	Mean patch time (min)		Estimated capture at apple-yeast		*G* statistic for goodness-of-fit
	AY	M	*N*	% at AY	
AY	13.4	12.9	75	49.1	12.91*
M	9.8	15.2	58	39.3	6.09*
NV	9.9	8.4	38	45.9	0.85

[a] Also shown is estimated frequency of captures on apple-yeast and mushroom baits assuming that experience affects patch residence time only. Also shown is *G* statistic for goodness-of-fit test between estimated distribution and that observed for females experienced with *Drosophila melanogaster* and released on day 1 (see Table 4). *G* values followed by an asterisk are significant at $P < 0.05$. AY = apple-yeast. M = mushroom. NV = naive.

TABLE 6. FREQUENCY OF CAPTURES AT APPLE-YEAST AND MUSHROOM BAITS ACCORDING TO TYPE OF EXPERIENCE[a]

Type of experience	Days between experience and capture	Captured on (N)	
		Apple-Yeast	Mushroom
Apple-yeast	6.9 (±0.39)	12	2
Mushroom	7.7 (±0.53)	13	11
Naive	8.1 (±1.20)	3	4

[a] Data includes captures made on days subsequent to the day of release. Also shown is the average number of days (±1 SE) between experience in the laboratory (if any) and capture in the field.

tured on the day they were released or else not at all, a small proportion of females were captured on subsequent days and some were captured as long as two weeks after their initial experience in the laboratory. The frequency of captures at apple-yeast and mushroom baits for these females is presented in Table 6. Females originally experienced with mushroom substrate, like naive females, were captured at apple-yeast and mushroom baits in approximately equal frequencies. Females originally experienced with apple-yeast substrate, by contrast, were captured almost exclusively at apple-yeast bait. It thus appears that apple-yeast-experienced females retained what they had learned even though an average of seven days had elapsed since their experience in the laboratory. Due perhaps to the low catch of apple-yeast-experienced females at mushroom baits ($N = 2$), the difference between apple-yeast-experienced and mushroom-experienced females was not significant (G test with Yates' correction, $P > 0.1$).

DISCUSSION

Most "experienced" *L. heterotoma* females probably laid eggs prior to release, while naive ones did not. Effects of experience on percent capture and travel time could conceivably reflect a physiological change contingent upon egg deposition but unrelated to learning. Females that failed to lay any eggs at all (i.e., naive females) might, for example, simply tend to disperse from the point of release and so be less likely to be captured in the immediate vicinity than females that recently laid eggs (i.e., experienced females). Such an interpretation, while plausible, is inconsistent with laboratory analyses of movement paths by female *L. heterotoma*. These analyses indicate clearly that females learn to orient upwind in a plume of a specific odor associated with previous oviposition. Such associative learning should increase the tendency for females to find a microhabitat in the manner observed in the field.

Moreover, it would be difficult to explain the tendency for females to choose the specific microhabitat associated with previous oviposition (Table 4) as anything except learning. We are confident therefore that our parasitoids were actually receiving and storing information about their semiochemical environment and adopting behavior consistent with that information. This inference is supported by a wealth of laboratory data on odor learning in this and related eucoilid species (Vet, 1988, and references within).

Our results also provide support on two counts for the notion that experienced individuals forage more effectively for essential resources than inexperienced ones, so long as those resources remain available. First, experience increased the likelihood that a bait would be found over the course of our censuses. Second, experience reduced the time required to find a bait (i.e., travel time).

Is the reduction in travel time with experience necessarily realized as an increase in the reproductive success of foraging wasps in nature? Does the increased likelihood of finding a microhabitat over the course of a day confer increased reproductive success? The answers to both questions are uncertain. Field releases by Driessen and colleagues (in preparation) suggest that females of a related species, *L. clavipes*, find at most a few host microhabitats over their lifetimes. Whether or not such circumstances are representative of *L. heterotoma* is unknown. If they are, learning to move more effectively upwind in an odor plume would confer an advantage in fitness only if females learned rather rapidly. In fact, *L. heterotoma* is capable of extremely rapid learning. In olfactometer assays, females show a dramatic improvement in odor response after a single oviposition or even a single contact with host kairomone (Vet, 1988; Vet and Groenewold, 1990). While it seems likely that females benefit by their experience, knowing how often individual females move from patch to patch is obviously critical to our arguments and warrants further attention.

Also deserving of further study is the extent to which *L. heterotoma's* environment is predictable between and within generations. Several observations suggest that *L. heterotoma's* environment is unpredictable, at least over successive generations. First, an adult female rarely breeds in the same patch in which it developed as a larva (Driessen, personal observations). Second, both *Drosophila* host species and their associated microhabitats are notoriously ephemeral in time and space (Shorrocks and Charlesworth, 1980; Shorrocks, 1982). In short, an emerging adult female parasitoid must disperse in search of oviposition sites and, when it does, may well encounter a different suite of microhabitat types than did its mother.

Other observations suggest that the environment is predictable, at least from the point of view of an individual female within a given generation. First, adult *L. heterotoma* are short-lived (ca. 11 days; Janssen 1989) and any changes that would render the environment unpredictable to an individual would have

to occur over a relatively brief period of time. Second, *Drosophila* microhabitats (i.e., host patches) are almost certainly distributed themselves as patches. If travel times between microhabitats within a patch are long (as they seem to be; Driessen et al., in preparation) and travel times between patches even longer, individuals may spend all or most of their brief lifetimes within a patch of one microhabitat type. Taken together, these observations suggest that *L. heterotoma* meets the conditions set forth by Stephens (1990) for the evolution of learning.

Regardless of the efficacy of odor learning in natural ecosystems, our results may have significance for biological control in agroecosystems where, because the vegetation is both dense and homogeneous, parasitoids attacking insect pests must commonly move between host microhabitats (i.e., plants) of like type. Although evidence that parasitoids learn under laboratory conditions abounds, only one other study to our knowledge (see Lewis and Martin, 1990) has demonstrated that experience influences parasitoid foraging behavior on a spatial scale larger than that of a small cage or an olfactometer. Now that we know that experience can markedly improve foraging effectiveness in an open habitat similar to a glasshouse or agricultural plot, we can ask if improvements in effectiveness exceed a variety of economic costs associated with "training" wasps to be more efficient foragers (see Vet and Groenewold, 1990). At present, we cannot state with certainty that conditioning parasitoids prior to release will improve the commercial efficacy of biological control, but surely a possible increase of 100% in parasitoid foraging efficiency (cf. Table 1) offers incentive for exploring this possibility.

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PROSPECTS OF ANTIFEEDANT APPROACH TO PEST CONTROL—A CRITICAL REVIEW

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Abstract—The increasing efforts to develop environmentally safer pest control methods have attracted the attention of many authors towards the use of antifeedants. This review is a critical survey of the most important publications issued during the 1980s, especially dealing with sensory physiological and behavioral studies, structure–activity aspects, screening methods, sources of antifeedants, and aspects of practical application. Important but neglected areas of research as well as future tasks are stressed.

Key Words—antifeedant, feeding inhibitor, deterrent, allelochemical, electrophysiology, behavior, integrated pest management, screening, field trials, systemic action, persistence, resistance, structure–activity relation, habituation, toxicity, third trophic level.

INTRODUCTION

As a consequence of the overall striving for environmentally safe pest control methods that can become part of sustainable agriculture, research on insect feeding deterrents, i.e., antifeedants, has attracted increasing attention during the last decade. At the beginning of the 1980s several papers (Schoonhoven, 1982; Bernays, 1983; Jermy, 1983) reviewed the results obtained that far. The following critical review attempts to outline how the scientific background and the implementation of the antifeedant approach to pest control has further developed during the 1980s especially stressing the main lines of research that seem to be most important in the near future.

The number of relevant publications issued during the last decade is very

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significant. Because of space limits, many details cannot be discussed here and the review is restricted to insect pests of field, garden, and glasshouse crops.

For terminological reasons, discussed by Schoonhoven (1982), the terms "antifeedant" or "feeding deterrent" will be used here.

SENSORY AND BEHAVIORAL BACKGROUND

Chemosensory Aspects. Surveying the literature on the sensory effects of antifeedants, Schoonhoven (1982) concluded that feeding deterrents evoke at least five different types of stimulating or inhibiting effects on the insects' chemoreceptors. The complexity of the neurophysiological processes involved has been further proved by more recent findings (Frazier, 1986; Bernays and Chapman, 1987; Dethier, 1987; Schoonhoven, 1987).

Studying the behavioral and neurophysiological effects of a series of clerodane diterpenoids on the larvae of five lepidopteran species, Blaney et al. (1988) proposed that the mechanism of excitation in case of deterrents may not be of the enzyme–substrate type. An alternative explanation would be that the deterrent compounds act upon the receptor membrane in a rather unspecific way. Blaney et al. (1988) observed significant differences in the receptor responses among the larvae of closely related lepidopteran species, a finding that further complicates the picture. Schoonhoven and Yan Fu-Shun (1989) compared the receptor responses to three aldehyde sesquiterpenoid antifeedants and found that small variations in the molecular structure evoked different responses.

Analyzing the effect of antifeedants on normal chemoreceptor activities in lepidopteran larvae, Frazier (1986) and Schoonhoven and Yan Fu-Shun (1989) have shown that, e.g., sesquiterpene antifeedants stimulate the so-called deterrent cell and decrease the sensitivity of most or all phagostimulant receptors to several different phagostimulants. The latter authors suggest that possibly the blocking of sulfhydryl groups in receptor membrane proteins is the underlying mechanism. The pattern of the sensory input evoked by antifeedants seems to be species specific and more or less compound specific.

To my knowledge, until now no experiments have been carried out to reveal how an antifeedant compound affects the sensory input evoked by the host plant's sap. Electrophysiological investigations using mixtures of antifeedants and plant saps would answer this question.

The complexity of deterrent perception makes it unlikely that the screening of compounds for antifeedant activity will be possible by electrophysiological methods. This is unfortunate, because such a method would enable the quick prescreening of many candidate compounds.

Habituation. One of the important problems concerning practical appli-

cation of antifeedants is habituation to them. Earlier studies have demonstrated habituation, and it was proposed that habituation is more likely to occur with polyphagous insects than with oligophagous ones (see Jermy, 1987, for references).

Raffa and Frazier (1988) developed a model for quantifying behavioral habituation to antifeedants. It allows for measurement of habituation as a function of all contributing factors, and it enables a quantitative comparison of antifeedant compounds, insect species, and host-plant species that is indispensable for field trials and application. Based on this model, the authors proposed also that the terms "absolute" and "relative" antifeedant (Munakata, 1977) should be used with regard to a particular concentration, because the effect is most likely always dose-dependent.

To my knowledge, no detailed research has been carried out on habituation under field conditions, or on the behavior of the insects on antifeedant-treated plants in nature. Only Szentesi (1981) reported that Colorado potato beetle females laid more eggs on antifeedant-treated potato plants than on untreated ones.

Mode of Feeding. The effect of an antifeedant on the mode of feeding has been observed by Saxena and Khan (1985) on the cicadellid *Nephotettix virescens*. Electronic monitoring of feeding has shown that the normal feeding of the insect on phloem became erratic and partly changed to feeding on xylem when the food was rice plants growing in soil treated with neem seed cake. (Neem antifeedants are taken up by the plants.) The reduced infection of the treated plants with tungo viruses, which are phloem specific and are transmitted by this cicadellid, was attributed to the disruption of phloem feeding.

COMBINED BEHAVIORAL AND PHYSIOLOGICAL EFFECTS

Clearly, compounds with combined behavioral (antifeedant) and toxic effects would more likely warrant successful practical use than compounds with only behavioral activity. Testing for combined action may need special experimental protocols, because the physiological (e.g., morphogenetic) effects of feeding inhibition, i.e., starvation itself, are not always easily distinguishable (Sláma, 1978).

Until now, most information on a combined activity is available on azadirachtin and various neem extracts and products. Their effects on the insects' neuroendocrine and reproductive systems as well as on enzyme activity have been further proved on various pest species during recent years (see chapter "Mode of Action" in Schmutterer and Ascher, 1987, and references therein). A dual antifeedant and antihormone action was found also with sesquiterpene lactones (Nawrot et al., 1984).

The significant advantage of a dual action was demonstrated by Kraus et al. (1987); high concentrations of azadirachtin had an antifeedant effect, while at low concentrations enough azadirachtin was ingested to result in growth regulation. It is most likely that the overall favorable experience gained with neem products is largely due to this.

However, a very broad toxic effect on the insects may reduce the target specificity of the antifeedant because it may affect entomophagous insects also.

STRUCTURE–ACTIVITY ASPECTS

As a continuation of earlier attempts, several authors have dealt with the structure–activity problem during the last few years. For example, Yano (1986) reported that a hyperconjugatable triple bond between C atoms in the side chain of phenylalkynes was associated with antifeedant activity for *Pieris rapae crucivora* larvae. According to Hassanali and Bentley (1987), at least three regions in the limonoid skeleton may be involved in eliciting deterrence in the larvae of three lepidopteran species. Blaney et al. (1988) proposed that a side chain at C-9 on the decalin ring of clerodane diterpenoids was important for feeding inhibition in the larvae of five lepidopteran species tested, but negative findings also were reported, e.g., Lidert et al. (1987) and Polonsky et al. (1989) found no obvious correlation between structure and antifeedant activity in quassinoids.

As regards structure–activity studies, it has to be kept in mind that the validity of relations found will most likely always be restricted to the insect species and to the group of compounds tested. The earlier statement that oligophagous insects are more prone to feeding inhibition has been supported by recent findings (e.g., Hassanali and Bentley, 1987; Blaney et al., 1988). This already indicates the great interspecific differences in the susceptibility to a given antifeedant compound. Nevertheless, structure–activity studies may shed some light on the molecular processes occurring at the receptor membrane level of a given insect species and thus may help in the synthesis of the most active molecules within a group of compounds for particular insect species.

SCREENING

The screening of substances for antifeedant activity has been carried out almost exclusively in laboratory tests during the last years using methods reviewed by Schoonhoven (1982) and Chansigaud and Strebler (1986). Most authors used short-term, often two-choice assays and did not carry out direct observations on the behavior of the test insects.

By a long series of experiments using a single antifeedant and one insect

species, Bernays and Wege (1987) demonstrated that experiments need repetition even when significance levels in one experiment indicate an unequivocal response, because otherwise variations of the effect can remain undetected.

Usher et al. (1988) compared the results of short-term and long-term antifeedant tests and concluded that only long-term assays are adequate to screen chemicals for possible practical use, while the results of short-term behavioral observations may reveal important details of the insects' behavior in a diverse plant community. Long-term assays may indicate the possibility of habituation that may ab ovo exclude practical use of antifeedants. For this reason, Raffa and Frazier's (1988) model of quantifying habituation should be considered in future screening assays. Morphogenetic or other toxic effects can be observed only in long-term tests.

One of the weak points of investigations into the occurrence of antifeedant substances is that in most cases only one test insect species is used. The majority of tests reported in the literature were carried out on the larvae of the lepidopterans *Spodoptera* spp., *Heliothis* spp., *Pieris* spp., and on locusts *Locusta migratoria* and *Schistocerca gregaria* than can be reared in laboratories. In most papers the structure of the compound(s) found to be active in the laboratory test on one or a few insect species is reported, but no further attempts are made to find out what other species the substance is active against, how stable it is under natural conditions, etc. Often the described compounds are not available or not in suitable quantities for further studies. It is, therefore, difficult to judge what proportion of the numerous antifeedant compounds described so far can be regarded as promising for practical application.

The substrate used for screening may be of great importance. Various authors use agar, agar cellulose, filter paper, glass fiber disks, styropore lamellae, artificial diets, etc. (see Schoonhoven, 1982, for references). However, the insects' response to the same chemical may differ depending on the stimulus situation. For example, *Schistocerca gregaria* showed habituation to nicotine–hydrogen–tartrate when it was provided on leaf disks, but showed aversion learning when the substrate was sucrose-impregnated glass fiber disks (Jermy et al., 1982).

Similarly, screening chemicals by incorporating them into artificial diets may be of questionable validity, because the quality of the plant's surface that is first encountered by the insect largely determines acceptance or avoidance (Chapman and Bernays, 1989). Therefore, a compound may be nondeterrent when mixed into the diet but may be effective on the leaf surface and vice versa.

Phytotoxicity may not manifest itself in short-term leaf-disk tests. This author repeatedly observed that phytotoxic effects appeared on leaf disks only after 20–24 hr (Jermy, unpublished). In such cases the question remains open whether feeding inhibition was caused by the direct behavioral effect of the chemical or by the changes elicited in the chemistry of the plant by the chem-

ical. In this connection, Polonsky et al. (1989) pointed out that phytotoxicity and antifeedant effect are not necessarily correlated, e.g., sergeolide and ailanthinone were phytotoxic but did not deter aphids from settling, whereas quassin was an effective antifeedant, but was not detectably phytotoxic. Meisner et al. (1987a) reported that several herbicides that are recommended for weed killing in alfalfa, showed antifeedant activity on the larvae of *Spodoptera littoralis* when sprayed on this crop. These authors did not study the mode of action, but it cannot be excluded that changes caused by herbicides in the plants were also or even mainly involved. This is the more likely since it has been shown that herbicides may alter the chemistry of the crops in which they are used (e.g., Edwards et al., 1983). Compounds acting as feeding deterrents in this way can be called "indirect antifeedants." Clearly, it would be important to screen chemicals from this point of view also.

Redfern et al. (1984) observed phytotoxicity of neem extracts on greenhouse-grown sweetcorn plants. This may suggest that the high activity of neem products is partly due to an "indirect antifeedant" effect. The reduction of the yield caused by neem extracts in combination with piperonyl butoxyde on potato (Zehnder and Warthen, 1988) also may indicate a physiological effect on the plant.

The increasing gap between the vast numbers of papers reporting on successful laboratory tests and the scarce results of field experiments is due mainly to the unsuitability of testing procedures. It is clear that in order to find practical antifeedants, screening methods should be used that best simulate field conditions. Thus, only long-term screening on whole plants can provide suitable information for subsequent field trials. However, the quality of the plants also is relevant. Since greenhouse-grown plants may differ significantly from field-grown ones in their chemistry and tissue structure, only field-grown plants can provide reliable results. Although such plants can be used only during the growing season, this disadvantage is largely counterbalanced by the wealth of information received by such tests concerning antifeedant and/or morphogenetic effects, persistence of activity, phytotoxic effect, and systemic action.

The choice of suitable plants for screening is important also because Raffa (1987) found that the deterrent effect of azadirachtin was strongly influenced by the plant species used in the trials (see below).

FIELD TRIALS

Large-scale field or green house trials are still very scarce, although they are indispensable before any practical use can be proposed (Bernays, 1983). The Colorado potato beetle was the first pest against which antifeedants, especially copper-based fungicides, were proved to be effective on large-scale appli-

cation decades ago. (See the review by Szentesi and Jermy, 1985.) These findings were further proved by recent successful pest management programs against this pest (Hare, 1984; Hare and Moore, 1988).

Among the antifeedant plant semiochemicals, the most extensive field trials have been carried out with neem products, mostly due to broad international cooperation. For example, nine papers at the Third International Neem Conference (Schmutterer and Ascher, 1987) reported on successful field applications. The results were often comparable with the effect of commercial insecticides.

SOURCES OF ANTIFEEDANTS

Plants. Regarding the narrow host-plant specialization of most phytophagous insect species that is due largely to the presence of feeding inhibitory substances in the non-host-plant species, it is logical that most attempts for finding new antifeedants have been directed to various plant species during the last years. Space does not allow reference to the vast number of publications dealing with screening of plant extracts on various insect species. According to Ahmed et al. (1984), there are about 2000 plant species purported to possess pest-control properties, and Yang and Tang (1988) recently reported that in China different parts or extracts of 276 plant species are used as pesticides. Most likely a large part of these effects is due to deterrents.

So far mostly tropical plant species have been successfully tested for their antifeedant contents. This is due partly to the increasing attempts to replace synthetic insecticides with less expensive and locally available pest control means, especially in the tropics. However, it is obvious that temperate-zone plants also contain suitable compounds, as has been shown, e.g., by Reed et al. (1981) and Abivardi and Benz (1984). The neem tree (*Azadirachta indica*) is, in this respect, the most thoroughly investigated plant species. The imposing volumes of the Neem conferences (Schmutterer et al., 1981; Schmutterer and Ascher, 1984, 1987) prove how rapidly and how far research can develop if active international cooperation is involved.

That plants may contain several compounds with antifeedant activity has been further confirmed recently. For example, Matsumoto and Sei (1987) and Yan Fu-Shun et al. (1990) found several components in *Ginkgo biloba* inhibiting feeding of *Pieris brassicae* and *P. rapae crucivora*. Extensive studies on the extracts of *Azadirachta indica* and *Melia azedarach* have revealed the structural diversity of antifeedant compounds in these plants (Kraus et al., 1987; Sankaran et al., 1987).

It is generally known, but still often not duly considered, that plant chemistry may show significant intraspecific variation due to genetic (population),

climatologic, meteorologic, nutritional, and cultural factors, among others. It is unfortunate that in many publications the ecological conditions under which the plants tested were grown are not given. Intraspecific variation may become an especially intriguing problem when large-scale production of an antifeedant of plant origin should be initiated. As a well-documented example, the variability of antifeedant activity of neem extracts from different geographic regions and ecological conditions should be mentioned (Ermel et al., 1984; Singh, 1987).

Herbicides. Meisner et al. (1987a) found that the herbicide pronamide, which is specifically recommended for use on alfalfa, as well as propham and simazine acted as antifeedants against *Spodoptera littoralis* larvae on alfalfa. The mode of action of these substances has not been studied in detail. Although these herbicides are not phytotoxic to alfalfa, it cannot be excluded that they acted as "indirect antifeedants" (see above). It has been shown that, e.g., propham formed sugar conjugates in the alfalfa plant (Zurqiyah et al., 1976).

Fungicides. As mentioned above, copper-based fungicides proved to be very effective antifeedants against the Colorado potato beetle. Organotin compounds also were found to inhibit feeding of many insect pests (see the bibliography by Ascher, 1979).

Miticides. In field trials, two properly timed applications of the miticide dicofol, by its antifeedant effect, prevented defoliation of the potato by the Colorado potato beetle from exceeding 20% (Walgenbach and Wyman, 1987).

Other Sources. Taking into consideration the chemosensory background of feeding behavior in phytophagous insects, it is not surprising that seemingly strange sources of antifeedants have been found. For example, Saleh et al. (1984) extracted an antifeedant azulene component from the brown alga *Dictyota dichotoma.* Rowan and Tapper (1989) isolated the antifeedant peramin from the fungus *Acremonium lolii.*

These extreme examples indicate that there must be many undiscovered sources of new antifeedants. As has been emphasized earlier (Jermy, 1983), more attention should be paid for screening by-products and waste materials of pharmaceutical and other organochemical and food industries, etc., which might turn out to be economically feasible new sources.

Built-in Antifeedants. In Bernays' (1983) opinion, breeding crops for insect resistance is more promising than the use of antifeedants. It seems that the rapid development of genetic engineering will soon enable us to combine both approaches by transferring the genes for the production of antifeedant allelochemicals from one plant species into another.

The gene for producing the compound leptine, which deters the Colorado potato beetle, has been successfully incorporated into the potato plant by fusing cells of wild plant species and commercial potato plants and growing the plants from cell culture (Sinden, 1988).

This question deserves considerable attention in future research.

ASPECTS OF PRACTICAL APPLICATION

Mammalian Toxicity. Since antifeedants act on the insects' behavior and are not necessarily toxic even to insects, it is theoretically possible to find antifeedants lacking any harmful effect to humans. It has been shown, for example, that mint hay remaining after vapor distillation of mint oil contained substances that inhibit feeding of the Colorado potato beetle (Jermy et al., 1981). Furthermore, quinine is a potent antifeedant against many phytophagous insects, while the amount prescribed as a medicine or taken in bitter soft drinks is many orders of magnitude greater than the residues that would remain in foodstuffs if quinine were used for pest control. Caffeine also was found to have an antifeedant effect (Nathanson, 1984). Some commercially used sweetening agents turned out to deter aphids (Nanayakkara et al., 1987).

It is unfortunate, however, that since Schoonhoven's (1982) review in which it was emphasized that (p. 66) "antifeedants of plant origin have hardly been investigated for toxicity in vertebrates," very few data have been published in this respect. As an exception, the product Margosan-O, a pesticide containing azadirachtin, can be mentioned. It is harmless to birds, fishes, rats, and it is not mutagenic (Larson, 1987).

Persistence. Resistance of antifeedants to degrading environmental effects is a prerequisite for practical application. The persistence of metal–organic antifeedants, such as copper and tin fungicides, is well established, while that of antifeedant plant allelochemicals is largely unknown.

In this respect, detailed investigations have been carried out only with neem products. For example, according to Stokes and Redfern (1982) azadirachtin lost antifeedant activity against *Spodoptera frugiperda* within 400 hr when exposed to sunlight. Ermel et al. (1987) showed that the azadirachtin content of neem kernel extract sprayed on bean plants degraded to 50% in 12 days in the greenhouse when irradiated with UV light daily for 12 hr. Neem oil deteriorated in rice plants in four days, but adding sunshields, such as carbon powder or liquid latex, prolonged its antifeedant activity (Saxena, 1987). Barnby et al. (1989) found that exposure of azadirachtin and three related compounds to ultraviolet radiation up to 200 hr did not affect their activity related to inhibition of pupation.

Because of its importance, much more investigation should be aimed at the problem of persistence. As a first step, small field experiments should be carried out with the promising compounds, followed by investigations into formulations of increasing persistence.

Systemic Action. This is a preferable condition, because it may enhance persistence and uniform distribution of the antifeedant within the plant, especially protecting new leaves, although an antifeedant on the surface may more effectively counterbalance the phagostimulatory effect of plant surface chemicals (Chapman and Bernays, 1989). The systemic action of neem extracts is

well documented and has been further proved (Larew, 1987), together with extracts of *Amoora ruhituka* (Meliaceae) and *Annona squamosa* (Annonaceae) (Islam, 1987). Uptake of neem ingredients and translocation in maize plants took about four days to reach effective concentrations in the plant against *Ostrinia nubilalis* larvae (Meisner et al., 1987b). The uptake of active substances by rice seedlings from the seeds treated with neem extracts has been shown by Abdul Kareem et al. (1989).

Further investigations into the systemic action of promising antifeedant compounds are needed.

Simple Substances or Mixtures? The effective defense of the nonhost plants must be due in many cases to a number of different chemicals acting in concert as deterrents (Jermy, 1983, and references therein). Furthermore, deterrent compounds belonging to the same chemical group may evoke different responses from the chemoreceptors (Schoonhoven and Yan Fu-Shun, 1989); thus, their combined effect is very likely more stable than that of single compounds. The successful application of neem extracts may be due at least partly to the multiplicity of deterrent compounds occurring in them (Saxena, 1987).

Zehnder and Warthen (1988) reported that addition of piperonyl butoxide to neem seed extracts enhanced toxic and antifeedant effects on larvae and adults of the Colorado potato beetle. [Piperonyl butoxide was itself an effective antifeedant against this pest at high dosage without toxic effect (Silcox and Shidiu, 1986)].

If several insect species are to be controlled by antifeedants on a crop, a mixture of compounds would more likely be effective because of the great interspecific variation in the response to a given compound. Comparative trials with simple antifeedant compounds and with their combinations, especially under field conditions, are indispensable.

Substrate Dependence of Antifeedant Effect. Since the relation of phagostimulatory and phagoinhibitory stimuli in the plants determines the degree of preference within the host-plant spectrum of a given insect species, it can be supposed a priori that an antifeedant compound would be more effective on a less preferred host plant than on a highly preferred one. Accordingly, Raffa (1987) found that equivalent concentrations of azadirachtin deterred the larvae of *Spodoptera frugiperda* less on cotton (highly preferred) relative to Lima beans (less preferred). However, Raffa (1987) reported that aristolochic acid strongly deterred feeding also on Lima beans, which may indicate a different effect of this compound on the chemoreceptors of the larvae. Copper-based fungicides were most effective antifeedants to the Colorado potato beetle on tomato, the least preferred host (Hare, 1984).

Resistance to Antifeedants. No investigations were carried out on the development of heritable resistance to antifeedants. As a matter of fact, it is

unlikely that, e.g., oligophagous insects could easily develop general resistance to feeding inhibitory substances, because this would result in rapid changes of their host-plant range, which is determined mainly by the occurrence of such substances in the nonhost plants. However, such changes are very rare events in nature. Nevertheless, development of resistance to antifeedants seems to be analogous to the appearance of insect strains that break nonpreference type of resistance in cultivars. Therefore, the permanent application of an antifeedant may result in the emergence of resistant (tolerant) insect strains. However, it cannot be predicted how broad such a resistance would be concerning the different molecular structures of possible antifeedant compounds.

Effect on Third Trophic Level. In principle, antifeedants do not harm members of the third trophic level. Furthermore, inhibition of feeding necessarily retards the herbivores' ontogenetic development, i.e., prolongs their exposure to natural enemies (Weseloh, 1984). Thus, antifeedants are ideal pest-control agents to combine with entomophages.

Unfortunately, up to the present very little has been done in this respect. Scattered data are available on the harmlessness of neem extracts on predators and parasites of insect pests (Saxena, 1987, and references therein). Such extracts were found also to be more toxic to phytophagous mites than to predatory ones (Mansour et al., 1987; Chiu, 1987).

Field experiments are especially necessary to evaluate the possibility of combining antifeedants with entomophages.

Toxicity to Bees. Schmutterer and Holst (1987) reported that neem seed extracts sprayed onto flowers caused some morphogenetic effects in very small honeybee colonies but not in large ones. Much more information is needed, especially in cases when a candidate antifeedant exerts also physiological effects.

Antifeedants as Stressors. Inhibition of feeding represents a strong stress on intensively feeding ontogenetic stages of phytophagous insects; thus, in principle, antifeedants must increase the susceptibility of the pests to pathogens and insecticides. Plant extracts as antifeedants potentiated the effect of *Bacillus thüringiensis* on the larvae of *Spodoptera littoralis* (Salama and Sharaby, 1988). In experiments with *Tribolium castaneum*, neem oil synergized malathion, quinalphos, and monocrotophos, and it antagonized pyrethrum and endosulfan, but did not affect dimethoate (Parmar and Dutta, 1986), indicating that there is no general trend in the combined action of the antifeedant and the various insecticides. This question also deserves much more attention.

Antifeedants and Integrated Pest Management. The high target specificity of many deterrent substances makes them ab ovo ideal for incorporation into integrated pest management programs. It is deplorable that, although this has been repeatedly stated by several authors, with the exception of the Colorado potato beetle (Szentesi and Jermy, 1985; Hare and Moore, 1988; and references

therein) and the use of neem products against some pests (Schmutterer and Ascher, 1984, 1987), no reliable large-scale field experiments have been carried out to prove the practicability of such approaches.

It is worth mentioning that cultural methods may enhance the accumulation of feeding inhibitory allelochemicals in plants. Mihaliak et al. (1987) have shown that the larvae of the noctuid *Pseudoplusia includens* consumed less from the leaves of *Heterotheca subaxillaris* (Asteraceae) grown under nitrate-limiting conditions because of increased concentration of volatile leaf terpenes.

CONCLUSIONS

Research carried out during the last decade has significantly extended our knowledge on antifeedants. However, there are several aspects that deserve more attention in the future in order to enhance implementation of this approach to pest control.

Further combined electrophysiological, behavioral, and structure–activity studies are necessary to reveal the possibility of planning more active molecules.

For screening new compounds, the preferred methods are those closest to field conditions.

Besides plant allelochemicals, new sources of antifeedants should be sought, e.g., by-products and waste materials of chemical and food industries.

Studies on the possible role of antifeedants as stressors that increase the susceptibility of insects to pathogens and insecticides should be extended.

Genetic engineering studies should shed light on the possibility of transferring the genes responsible for the production of antifeedant allelochemicals from one plant species into another.

Much more attention should be paid to the formulation of antifeedant compounds and to large-scale field trials with special emphasis on integrated pest-control approaches. Suitable pilot field trials would help to convince farmers of the practicality of antifeedants.

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BEHAVIORAL AND ELECTROPHYSIOLOGICAL STUDY OF ANTIFEEDANT MECHANISMS ASSOCIATED WITH POLYHYDROXY ALKALOIDS

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Abstract—Eleven polyhydroxy alkaloids of plant origin were tested for antifeedant effects against larvae of the lepidopterans *Spodoptera littoralis*, *Spodoptera frugiperda*, *Heliothis virescens*, and *Helicoverpa armigera*. Data from behavioral and electrophysiological investigations were correlated to reveal information on the mode of action of the antifeedants. The pyrrolidine DMDP was an effective antifeedant for all four species, whereas the piperidines fagomine and XZ-1 and the pyrrolizidine alexine were all ineffective as antifeedants. The activity of the pyrrolidines CYB-3 and DAB-1, the piperidines DNJ, DMJ, and BR1, and the bicyclic octahydroindolizine castanospermine varied among species. The investigation focuses on the structural similarities between some of the alkaloids and some common phagostimulatory sugars and illustrates a neural interaction involving the neurons that are differentially responsive to alkaloids and sugars. In *S. littoralis*, the neurons responding specifically to the alkaloids DMDP, DAB-1, and castanospermine and to the sugars fructose, sucrose, and glucose are more active when the compounds are applied singly than when an alkaloid and a sugar are applied together. The implications for the occurrence and functioning of different sugar receptor sites are discussed.

Key Words—*Spodoptera littoralis*, *Spodoptera exempta*, *Heliothis virescens*, *Helicoverpa armigera*, Lepidoptera, Noctuidae, polyhydroxy alkaloids, piperidine, pyrrolidine, castanospermine, swainsonine, alexine, fagomine, behavior, electrophysiology, receptor mechanisms.

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INTRODUCTION

Compounds that deter phytophagous insects from feeding could have a role in integrated pest management (see review by Jermy, 1990). For example, the polyhydroxy alkaloids, which are derivatives of piperidine, pyrrolidine, pyrroline, octahydroindolizine, and pyrrolizidine, have been shown to affect insect behavior and development (Blaney et al., 1984; Dreyer et al., 1985; Evans et al., 1985; Nash et al., 1986; Campbell et al., 1987; Fellows et al., 1989; Simmonds et al., 1989). Their ability to inhibit insect development and feeding could confer resistance against insect herbivory on plants in which they occur. So far, they have been isolated from five families of higher plants (Leguminosae, Polygonaceae, Euphobiaceae, Moraceae, Aspidiaceae) and many microorganisms (Fellows et al., 1989).

The majority of the compounds have been shown to inhibit glycosidases, although individually they have very different inhibitory properties (Fellows et al., 1989). Inhibition of digestive glycosidase enzymes would have the potential of affecting food utilization, and thus larval development. Using enzyme assays with homogenates of whole insect larvae, Fellows et al. (1989) showed that the disaccharidase activity in *Spodoptera littoralis* larvae differed in its susceptibility to inhibition by polyhydroxy alkaloids. The pyrrolidine 2(*R*), 5(*R*)-dihydroxymethyl-3(*R*), 4(*R*)-dihydroxypyrrolidine (DMDP) has been shown to be a competitive inhibitor of α-glucosidase in the larvae of the bruchid *Callosobruchus maculatus* F. (Evans et al., 1985), whereas castanospermine inhibits both α- and β-glucosidases, but its activity varies between insect species (Campbell et al., 1987). These studies indicated that the alkaloids show some specificity in their ability to inhibit enzymes.

Previous work has shown that castanospermine deters feeding in the pea aphid *Acyrthosiphon pisum* Harris (Dreyer et al., 1985) and that DMDP acts as an antifeedant against the locusts *Schistocerca gregaria* (Forsk.) and *Locusta migratoria* L. and against the caterpillars of *Spodoptera littoralis*, *Spodoptera exempta*, (Wlk.), and *Helicoverpa armigera* Hubner (Blaney et al., 1984; Fellows et al., 1989; Simmonds et al., 1989). The behavioral response of these caterpillars to polyhydroxy alkaloids has shown that they can discriminate between them, thus indicating that their sense organs respond differentially to stimulation with the compounds (Fellows et al., 1989; Simmonds et al., 1989).

Of the compounds used in this study, the piperidine and pyrrolidine polyhydroxy alkaloids have a simple monocyclic structure resembling monosaccharides with the ring oxygen replaced by nitrogen. The octahydroindolizines and pyrrolizidine have a bicyclic structure, but the configuration of the hydroxy substitutes on the rings is similar to that occurring in sugars. The way in which these compounds affect insects could be due to their structural similarity to sugar molecules.

The discriminating ability of larvae has been shown to be heavily dependent upon the activity of two particular types of neurons, often known as the "sugar neuron" and "deterrent neuron" (Schoonhoven, 1987). The polyhydroxy alkaloids have been shown to stimulate neurons responding to antifeedant compounds, but in addition, they also interfere with the normal functioning of neurons that respond to sugars (Simmonds et al., 1989). This latter effect may be due to their structural resemblance to simple sugars and may involve glucosidase inhibition. Glucosidases are known to be present in taste sensilla and have been implicated as the receptor molecules for sugars (Kijima et al., 1973; Amakawa et al., 1975; Kijima et al., 1977; Hansen and Wieczorek, 1981; Nakashima et al., 1982). These effects make polyhydroxy alkaloids interesting tools for the characterization of neuron receptors and for elucidating their specificity and role in neural coding.

In this paper we investigate the neural output generated by these compounds when they stimulate the maxillary styloconic sensilla of larvae belonging to four species of lepidopterans. These sensilla contain the principal taste neurons involved in food selection, and the responses of the sensilla will be related to the observed behavioral responses of the larvae to the alkaloids.

METHODS AND MATERIALS

Compounds

The polyhydroxy alkaloids (Figure 1) used in this study were: the pyrrolidines DMDP [2(*R*), 5(*R*)-dihydroxymethyl-3(*R*), 4(*R*)-dihydroxypyrrolidine], CYB-3 [2(*R*)-hydroxymethyl-3(*S*)-hydroxypyrrolidine], and DAB-1 [1,4-dideoxy-1,4-imino-D-arabinitol]; the piperidines deoxynojirimycin (DNJ) (1,5-dideoxy-1,5-imino-D-glucitol), deoxynojirimycin (DMJ) (1,5-dideoxy-1,5-imino-D-mannitol), fagomine (1,2,5-trideoxy-1,5-imino-D-arabinitol), fagomine glucoside (XZ-1) [1,2,5-trideoxy-4-*O*-(β-D-glucopyranosyl)-1,5-imino-D-arabinohexitol] and BR1 [2(*S*)-carboxy-3(*R*), 4(*R*), 5(*S*)-trihydroxypiperidine]; the bicyclic octahydroindolizines swainsonine {[1(*S*), 2(*R*), 8(*R*), 8a(*R*)]-1,2,8-trihydroxyoctahydro-indolizine} and castanospermine {[(1(*S*), 6(*S*), 7(*R*), 8a(*R*)]-1,6,7,8-tetrahydroxyoctahydroindolizine}; the pyrrolizidine alexine {[1(*R*), 2(*R*), 3(*R*), 7(*S*), 8(*S*)]-3-hydroxymethyl-1,2,7-trihydroxypyrrolizidine}.

The compounds were all extracted from plants (see Fellows et al., 1989).

Insects

Spodoptera littoralis (Boisd.), *Spodoptera frugiperda* (J.E. Smith), *Heliothis virescens* (F.), and *Helicoverpa armigera* (Hubner) were reared on a

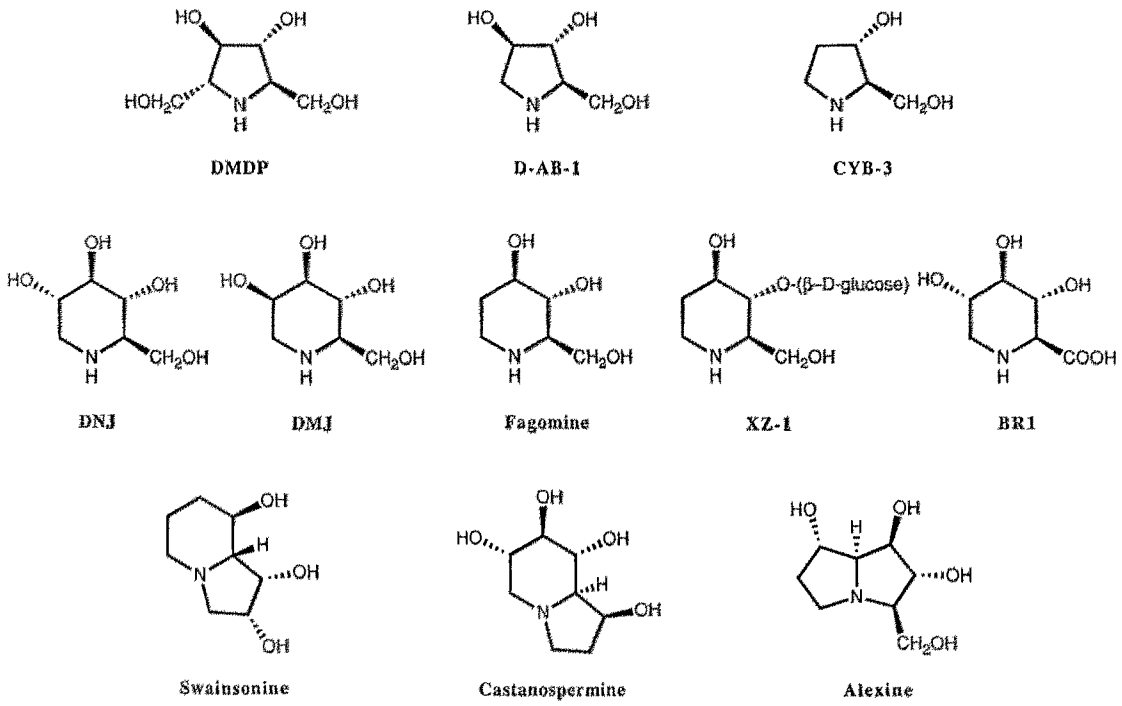


FIG. 1. The molecular structures of the compounds tested.

bean-based diet (Dimetry, 1976), at 26± °C in a 16:8 light–dark photoperiod. Larvae in the final stadium (6th) were used in the behavioral and electrophysiological bioassays. The larvae were at a stage in the stadium when feeding is vigorous (24–36 hr). Before being used in the bioassays, larvae were deprived of food for 2–4 hr.

Behavior

Choice Bioassay. The compounds were assessed for antifeedant activity by presenting them to larvae on glass fiber disks (GFD) (Whatman GF/A 2.1 cm). All disks were made palatable by the addition of 100 μl of a sucrose solution (50 mM). Control disks carried only sucrose, whereas 100 μl of a test alkaloid at one of three different concentrations (1, 5, and 10 mM) was added to treatment disks. The disks were then dried and weighed. For the bioassay, larvae were placed individually in a Petri dish with a control disk and a treatment disk. The bioassay terminated after the larvae had eaten approximately 50% of one of the disks or after 12 hr. The larvae were then removed, and the disks dried and reweighed. The antifeedant index $[(C - T)/(C + T)]\%$ was calculated, where C and T represent the amount eaten of the control and treatment disks, respectively. This index identifies both phagostimulants (− values) and antifeedants (+ values).

No-Choice Bioassay. The disks were prepared as in the choice bioassay. Only one concentration (5 mM) of the test compounds was used. In each test individual larvae were exposed to a single disk, either control or treatment. For statistical purposes, the experimental design used block treatments. A block consisted of a control and three to six test compounds that were tested against two species of larvae. Bioassays within a block were terminated when larvae exposed to control disks had consumed about 50% of these disks. The larvae were removed from the disks, which were dried and reweighed. The amount eaten was expressed as a percentage of the original weight of the disk. *S. littoralis* was exposed to all 11 compounds, whereas *S. frugiperda*, *H. armigera*, and *H. virescens* were only exposed to nine, five, and four of the compounds, respectively, because of their limited availability.

Behavioral Observations. In the no-choice bioassay, individual insects were observed continuously until they had made contact with the GFD, and discontinuously at 30-sec intervals thereafter, until they had completed their second meal. The behavior on initial contact was recorded as acceptance or rejection (Simpson et al., 1990), and the durations of both meals and the interval between them were recorded (Blaney et al., 1984).

Electrophysiology

Characterization of neural response. Test compounds were made up in sodium chloride (50 mM), which acted as an electrolyte. The maxillary stylo-

conic sensilla of larvae were stimulated with these solutions using a tip-recording technique (Hodgson et al., 1955; Blaney et al., 1984). Each sensillum was stimulated initially for 1 sec with the control solution (sodium chloride) then sequentially with two to three test compounds, each at three concentrations, with one stimulation of 1 sec for each concentration. The order in which the compounds were presented was random, and successive stimulations of one sensillum always were separated by at least 2 min. The response of a sensillum to test solutions was measured in terms of the total number of spikes generated in the sensillum during the first second of stimulation.

To help identify which of the four neurons in the lateral and medial styloconic sensilla were stimulated by the test solutions, five larvae of each species were exposed to the reference compounds to which the neurons are known to respond preferentially (Simmonds and Blaney, 1990) [lateral sensillum: neuron A = sodium chloride (100 mM), neuron B = alanine (5 mM), neuron C = sucrose (50 mM), neuron D = strychnine (1 mM); medial sensillum: neuron A = salicin (10 mM), neuron B = alanine (5 mM), neuron C = sucrose (50 mM), neuron D = canavanine (10 mM)]. The height, shape, and frequency of the action potential elicited from stimulating each sensilla with the reference compounds were compared with the profile of the action potentials elicited from responses of the same sensilla to five of the test solutions (5 mM). This was repeated with further batches of five larvae, using four to five different test compounds, until the electrophysiological responses of a species to all of the test compounds used in the behavioral choice test had been characterized.

Interactions with sugars. As a result of the data obtained in the behavioral and electrophysiological bioassays, the activity of DMDP, DAB-1, castanospermine, and swainsonine against larvae of *Spodoptera littoralis* was investigated further. Experiments were undertaken to investigate whether these compounds affected the ability of larvae to perceive sugars. The lateral styloconic sensilla of larvae were stimulated with a test compound in combination with one of the sugars (glucose, fructose, or sucrose) in two series of experiments, each using different larvae. In each series, larvae were first stimulated, at 2-min intervals, with sequentially increasing concentrations of one of the sugars, prior to stimulating with that sugar in combination with a test compound. In one series of experiments, the concentration of the test compound in the combination remained constant and the concentration of the sugar increased. In a second series, using fresh larvae, the concentration of the sugar remained constant and the concentration of the test compound increased. The concentrations of sugars and test compounds are given in Figure 5. The time between stimulations with the combination solutions was never less than 35 min. Preliminary experiments had shown that the time necessary for complete recovery of the response after a stimulation with a combination solution was greater than that required with solutions containing a single test compound.

In a further experiment, to investigate the effect of the test compounds on the subsequent response to sugars, the lateral sensilla of *Spodoptera littoralis* larvae were stimulated sequentially with a series of solutions: the electrolyte, sucrose, glucose, fructose, and one of the test compounds (DMDP, DAB-1, castanospermine, and swainsonine). After a pause of 45 min, the sequence of stimulations by electrolyte and sugar solutions only was repeated twice (see Figure 6a–d).

RESULTS

Behavior

Choice Bioassay. The antifeedant index for each compound has been plotted against the electrophysiological response to that compound (Figure 2a–d). The electrophysiological response represents the total number of impulses obtained from both the lateral and medial styloconic sensilla in the first second of stimulation. The data show that larvae of a given species respond differentially, both in behavioral and electrophysiological responses, to many of the compounds and that some responses are dose-dependent. However, the antifeedant activity of a particular compound varied between species. In order to reduce the complexity of Figure 2a–d, the variability associated with the behavioral and electrophysiological responses has been omitted. Overall, an ANOVA of the pooled data for all compounds and all species showed that the neural input was more variable than the behavioral response (antifeedant index), 40% of the total variability being associated with neural input and 27% with behavior. The principal effects shown by each species were as follows.

Spodoptera littoralis. Swainsonine, castanospermine, DAB-1, and DMDP were potent antifeedants. This behavioral response was dose-dependent and was associated with a high level of dose-dependent neural input. Although some of the piperidine compounds (fagomine, XZ-1) elicited a high neural input, they did not affect feeding significantly.

Spodoptera frugiperda. DNJ and DMDP were antifeedant at all three concentrations tested. The response to DMDP was not dose-dependent. Alexine and BR1 were antifeedant at the higher concentrations tested. The dose-dependent behavioral response to these two compounds was associated with dose-dependent neural input. Alexine elicited a greater neural response than BR1, although the latter was the more potent antifeedant. Fagomine and its glucoside XZ-1 were both phagostimulants. The behavioral response and neural input from these compounds were dose-dependent, increased phagostimulation being associated with increased neural input.

Heliothis virescens. Castanospermine and DMDP were the most potent

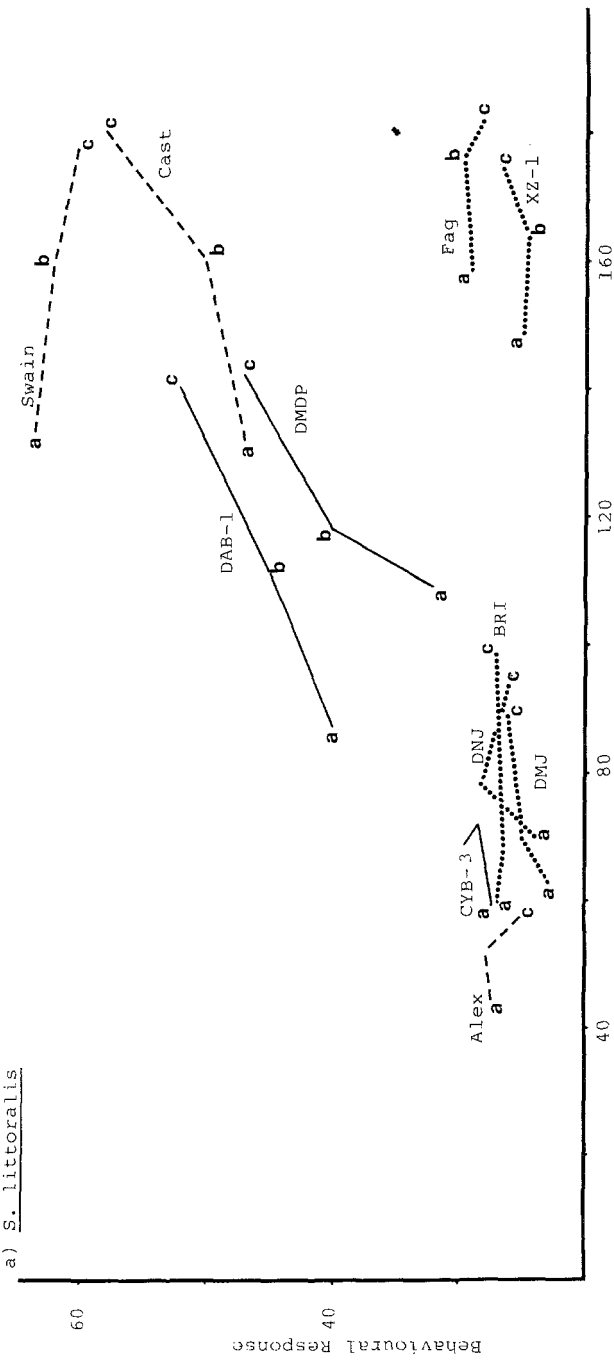


FIG. 2. The effect of test compounds at three concentrations (**a** = 1 mM, **b** = 5 mM, **c** = 10 mM) on the behavior (antifeedant index) and electrophysiological response (total impulses in first second from lateral and medial sensilla) of larvae of *S. littoralis* (a), *S. frugiperda* (b), *H. virescens* (c), and *H. armigera* (d). Pyrrolidines, piperidines, bicyclic octahydroindolizines, and pyrrolizidene compounds: see Fig. 1. (Alex = alexine, Cast = castanospermine, Fag = fagomine, Swain = swainsonine. (N = 5–20.)

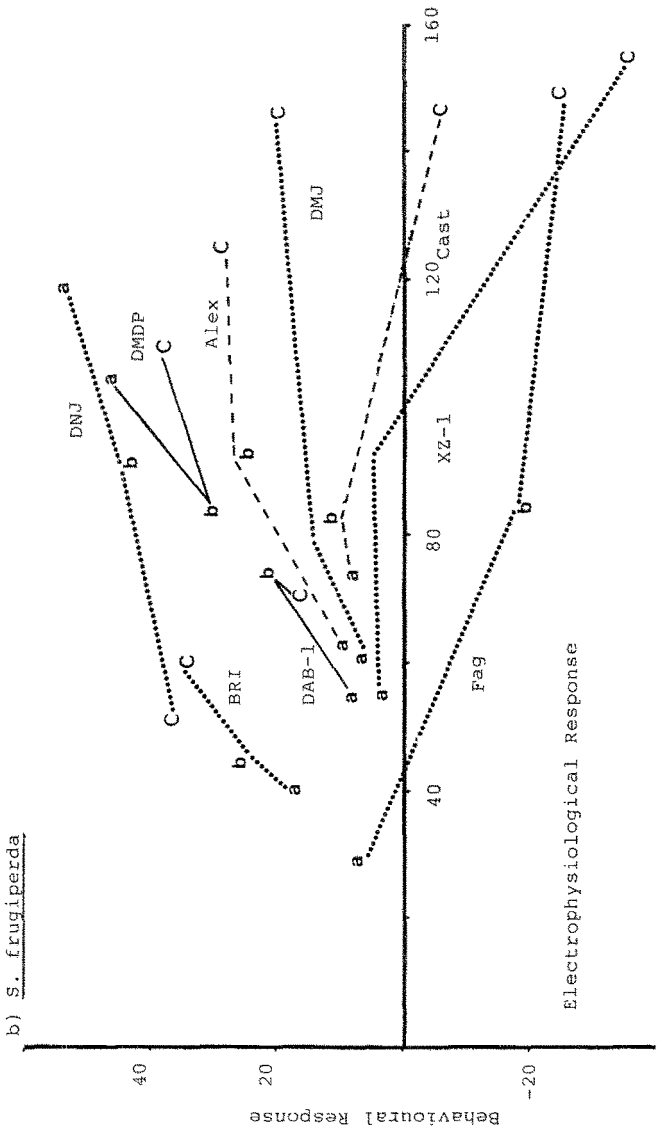


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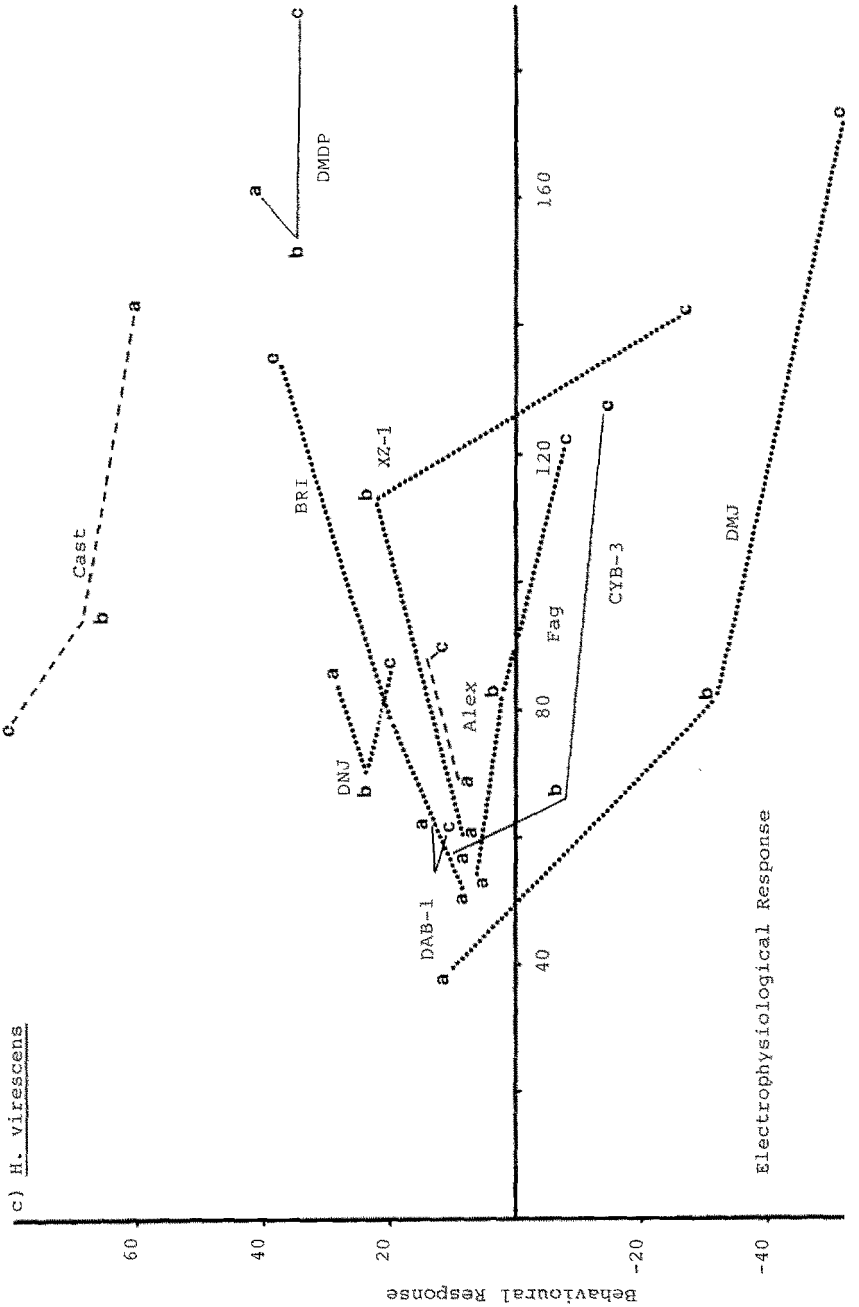


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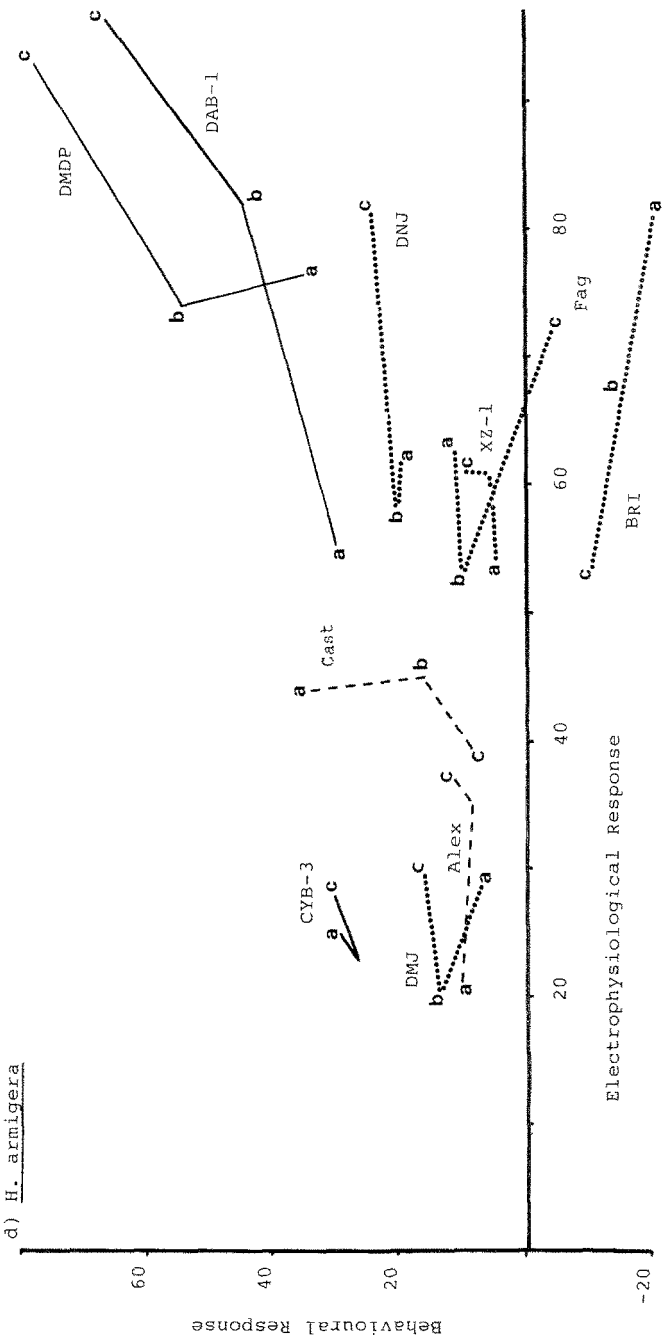


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antifeedant compounds. The behavioral and electrophysiological responses to castanospermine were dose-dependent, but the neural input decreased as the concentration of the compound increased. Thus, neural input was negatively associated with an increase in antifeedant activity. DMDP did not show a dose-dependent response. BR1 was the only piperidine compound to show antifeedant activity. DMJ was a potent phagostimulant, the behavioral response and neural input being dose-dependent.

Helicoverpa armigera. DMDP and DAB-1 were the most potent antifeedant compounds, with antifeedant activity increasing as neural input increased. Although the total neural input from the majority of the compounds varied, the resulting behavior, as indicated by the antifeedant index, was similar. In contrast with the other compounds, castanospermine produced a behavioral response that varied more than the neural input. Castanospermine was a more potent antifeedant at the lowest concentration than at the higher concentrations.

Previous studies undertaken with polyhydroxy alkaloids have shown that the behavioral response correlated well with the activity of a specific neuron in the lateral styloconic sensilla of *S. littoralis* (Fellows et al., 1989; Simmonds et al., 1989). Using the reference compounds (Simmonds and Blaney, 1990) to characterize the amplitude and spike profile of each neuron in each sensilla (for details about spike identification see Frazier and Hanson, 1986), we found that the majority of polyhydroxy alkaloids with antifeedant activity preferentially stimulated the neuron in the lateral sensillum responding to the reference compound strychnine (neuron D) (in Fellows et al., 1989, and Simmonds et al., 1989, this was called neuron C). The firing rate of neuron D in the lateral sensilla was determined and its response was plotted against the antifeedant index (Figure 3a–d).

Figure 3a–d show that the activity of neuron D in the lateral sensilla was significantly correlated with the antifeedant effect in all four species (*S. littoralis*, $r = 0.9417$, $N = 40$; *S. frugiperda*, $r = 0.7919$, $N = 35$; *H. armigera*, $r = 0.7934$, $N = 20$; *H. virescens*, $r = 0.7382$, $N = 20$; $P < 0.001$). However, the compounds stimulating this neuron varied with the species. DMDP, DAB-1, and alexine stimulated neuron D in all four species. Of these three compounds, DMDP elicited the greatest response, except with *S. littoralis*, where DAB-1 elicited the greatest response. Figure 3a shows that in *S. littoralis* neuron D was stimulated maximally by swainsonine and castanospermine and not at all by the piperidines DNJ, DMJ, and BR1; whereas in *S. frugiperda* (Figure 3b), of those compounds tested, neuron D was most sensitive to DMDP and DNJ, but castanospermine, fagomine, and XZ-1 did not stimulate this neuron. In *Heliothis virescens* (Figure 3c), castanospermine and DMDP elicited the greatest response from neuron D. The piperidines DMJ, fagomine, and XZ-

1 and the pyrrolidine CYB-3 did not stimulate neuron D in *H. virescens*, whereas BR1 was the only compound tested that did not stimulate neuron D in *H. armigera* (Figure 3d). In both *H. virescens* and *H. armigera*, the response of neuron D to castanospermine increased as the concentration of castanospermine decreased.

No-Choice Bioassay. The compounds shown to have antifeedant activity in the choice bioassay were also antifeedant in this bioassay. Compared to control larvae, a significantly higher proportion of larvae exposed to these antifeedant chemicals rejected the GFD on the initial contact, although feeding occurred on subsequent contacts (Table 1). However, the duration of the monitored meals or the intermeal interval was influenced by many of these compounds. The first meal taken by the larvae of all four species on the DMDP-treated GFD was not significantly different in duration from that taken by the larvae on the control GFD. However, the subsequent meal taken by *S. littoralis* and *H. virescens* larvae was significantly shorter than that taken by the control larvae, whereas with *S. frugiperda* and *H. armigera* the duration of the intermeal was greater than with the controls.

The antifeedant effect of DAB-1 on *S. littoralis* larvae resulted from the larvae taking meals of a significantly shorter duration than those taken by control larvae (Table 1). DAB-1 did not affect the duration of meals taken by *H. armigera*, although it did increase the duration of the intermeal interval.

Table 1 also shows that DNJ decreased the duration of the second observed meal taken by *S. frugiperda* and increased the intermeal duration. This could explain the low amount eaten. Swainsonine decreased the duration of both observed meals taken by larvae of *S. littoralis* and increased the intermeal interval, thus decreasing the amount eaten by the larvae. Castanospermine had a similar affect on *H. virescens*, whereas with *S. littoralis* it decreased the duration of the meals but did not affect the duration of the intermeal interval. Fagomine, XZ-1, and DMJ were the only alkaloids tested that stimulated feeding. *S. littoralis* was stimulated by fagomine and XZ-1 and *H. armigera* by DMJ.

The antifeedant activity shown by all the compounds in both behavioral bioassays is summarized in Table 2.

Interactions with Sugars. The mode of action of the active antifeedants DMDP, DAB-1, castanospermine, and swainsonine on *S. littoralis* was investigated in relation to their possible effects on the neural responses elicited by the sugars. When the alkaloids and the sugars were applied individually to the lateral sensilla at concentrations at which they are behaviorally effective, positive dose-dependent responses were obtained (Figure 4a,b). This figure shows the total neural activity in the lateral sensillum during the first second of stimulation. The response to the alkaloids was produced predominantly by neuron

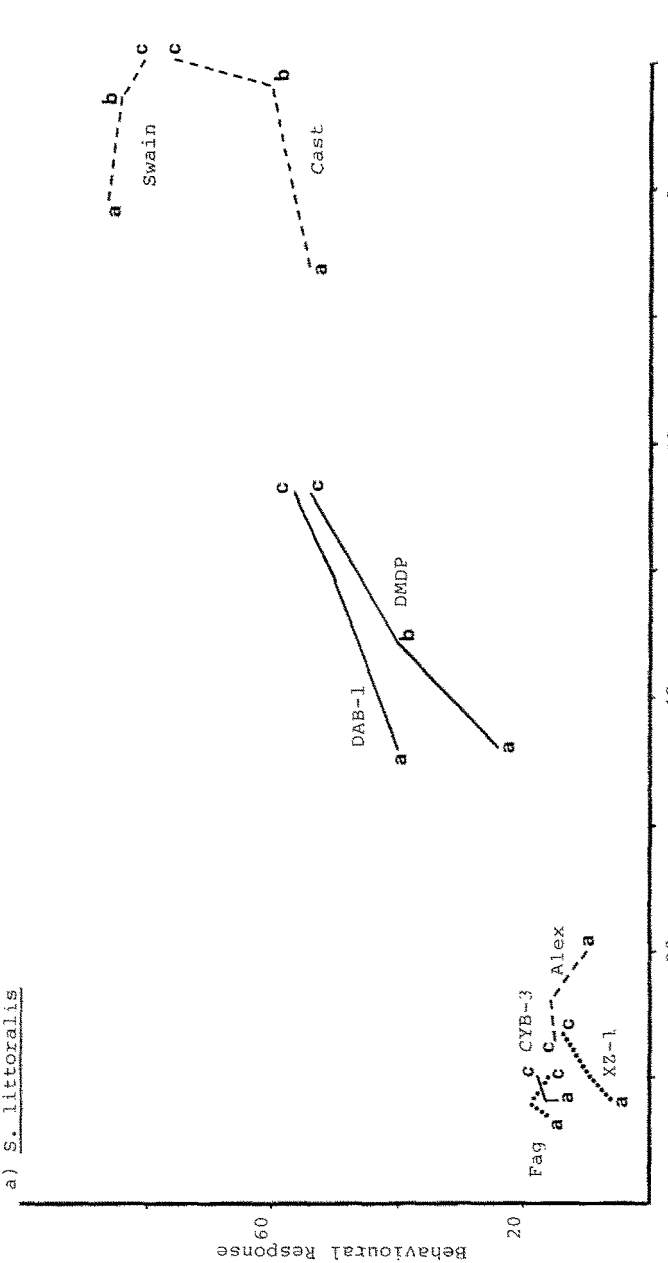


FIG. 3. The effect of test compounds on the behavior (antifeedant index) and electrophysiological response (impulses in first second from neuron D in lateral sensilla) of larvae of *S. littoralis* (a), *S. frugiperda* (b), *H. virescens* (c), and *H. armigera* (d). Compounds and concentrations as in Figures 1 and 2. ($N = 10–20$.)

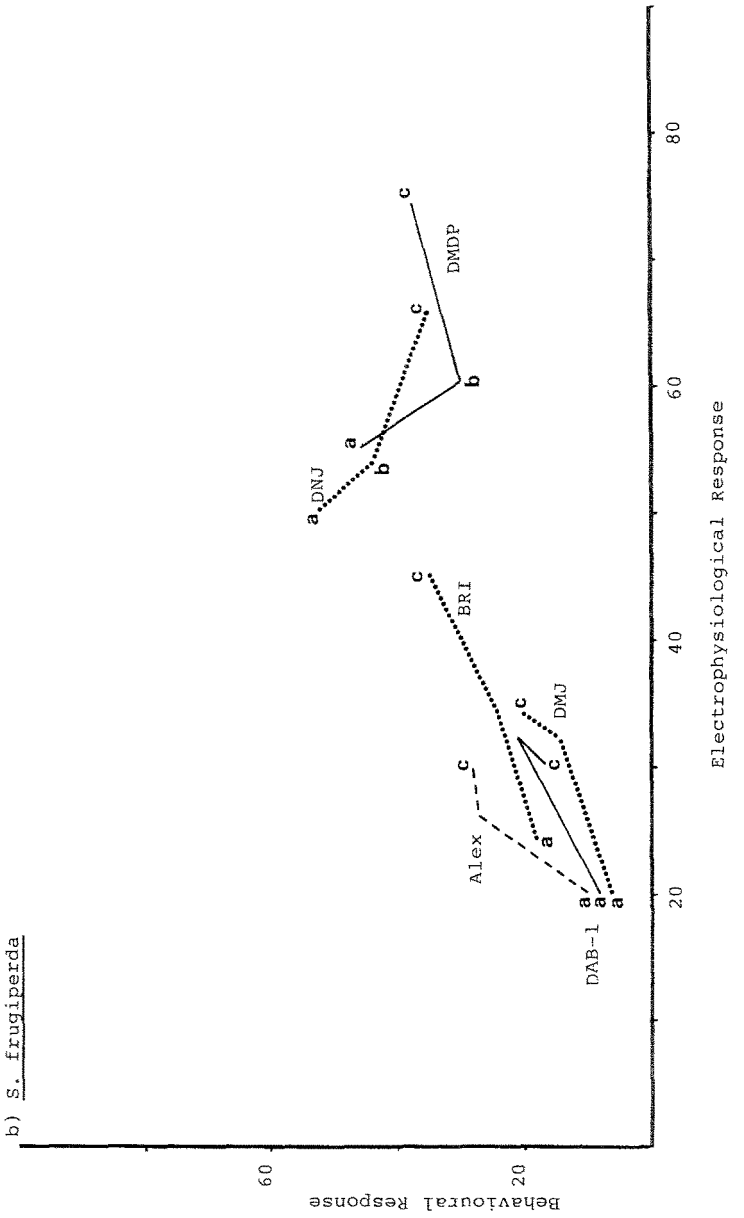


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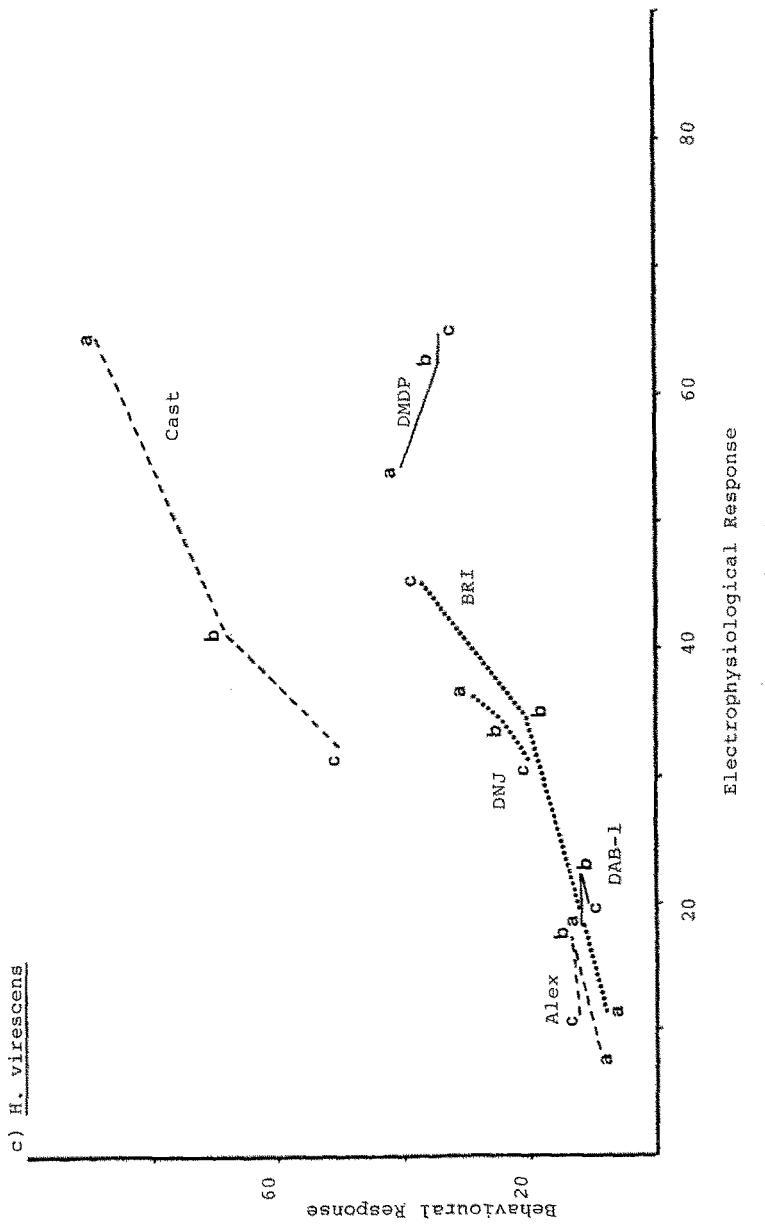


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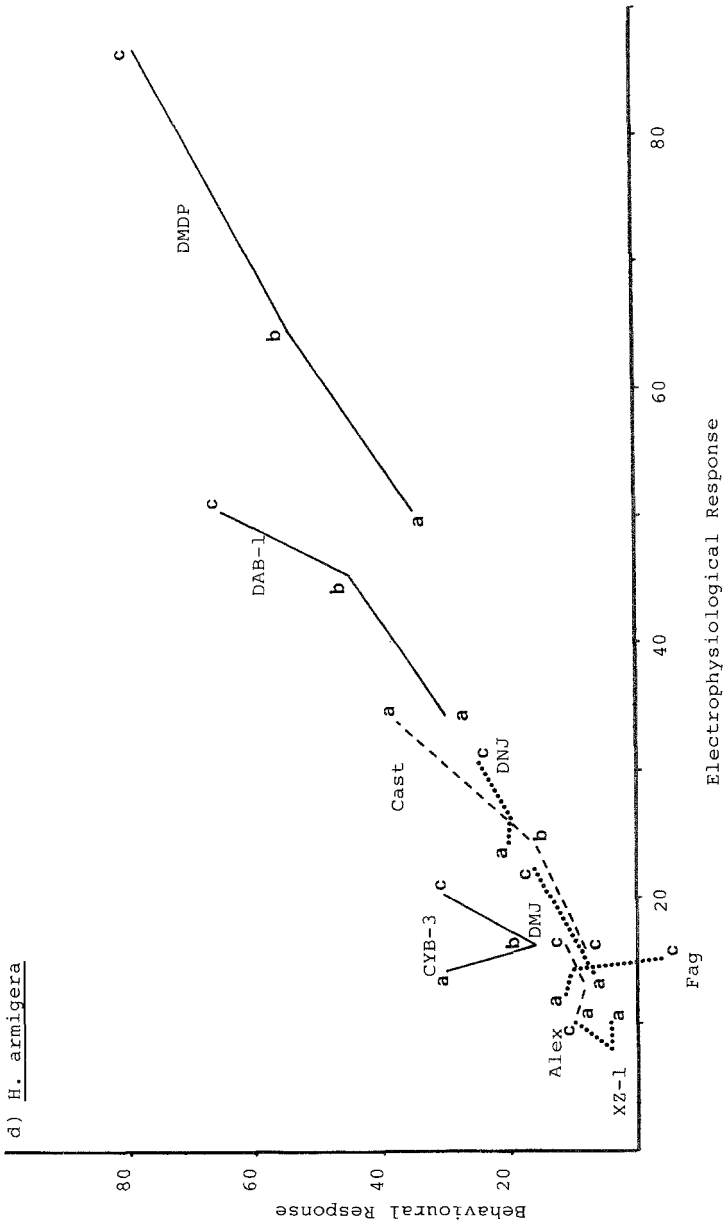


FIG. 3. Continued

TABLE 1. EFFECT OF TEST COMPOUNDS (5 mM) ON ASPECTS OF FEEDING BEHAVIOR IN NO-CHOICE BIOASSAY (N = 20)

Compounds	Rejection (%) 1st contact	Duration (sec) mean (SEM) 1st meal	2nd meal	Intermeal	Amount eaten (%) mean (SEM)
S.littoralis					
DMDP	75a	357 (145.9)	136 (45.7)[a]	265 (43.9)	10 (5.9)[a]
CYB-3	25	398 (120.9)	375 (165.9)	296 (76.9)	58 (5.6)
DAB-1	75a	165 (65.9)[a]	154 (125.9)[a]	344 (35.0)	12 (3.9)[a]
DNJ	30	355 (186.9)	357 (186.9)	343 (134.9)	46 (4.8)
DMJ	30	385 (186.4)	279 (137.9)	286 (197.8)	57 (3.8)
BRI	15	355 (244.9)	357 (186.5)	275 (322.9)	45 (4.9)
Fagomine	30	565 (344.8)[a]	377 (243.8)	233 (176.8)	65 (2.9)[a]
XZ-1	30	555 (267.7)[a]	344 (277.8)	242 (166.7)	65 (6.9)[a]
Swain.	75a	233 (155.8)[a]	233 (122.8)	676 (37.9)[a]	19 (3.9)[a]
Cast.	80a	166 (177.9)[a]	244 (43.9)[a]	355 (44.9)	23 (6.9)[a]
Alexine	15	377 (177.5)	264 (177.9)	366 (244.9)	56 (6.4)
Control (NaCl 50 mM)	15	386 (144.9)	366 (212.9)	248 (144.9)	53 (5.9)
S. frugiperda					
DMPD	75a	233 (127.9)	365 (186.8)	544 (186.8)[a]	25 (2.9)[a]
DAB-1	50a	273 (196.9)	288 (177.9)	355 (98.9)	56 (8.9)
DNJ	75a	283 (185.8)	277 (177.9)[a]	466 (187.9)	32 (5.9)[a]
DMJ	15	236 (185.2)	352 (195.9)	364 (199.4)	54 (5.9)
BRI	30	296 (186.7)	386 (166.7)	432 (74.9)	48 (2.9)
Fagomine	15	455 (244.9)[a]	366 (38.9)	377 (24.9)	49 (7.9)
XZ-1	15	299 (211.9)	475 (233.8)	379 (85.9)	48 (12.9)
Cast.	30	299 (188.6)	366 (186.5)	422 (122.9)	54 (8.3)
Alexine	30	312 (213.9)	362 (185.3)	533 (175.8)	52 (5.9)
Control (NaCl 50 mM)	30	228 (153.9)	361 (153.7)	352 (134.2)	51 (3.5)
H. virescens					
DMDP	50a	212 (121.9)	127 (61.9)[a]	266 (165.7)	36 (4.9)[a]
DNJ	50a	354 (108.6)	256 (106.9)	326 (144.6)	42 (5.9)
DMJ	10	436 (127.5)[a]	285 (123.9)	218 (101.3)[a]	64 (8.8)[a]
Cast.	75a	187 (93.9)[a]	122 (84.6)[a]	418 (265.9)[a]	16 (6.8)[a]
Control (NaCL 50 mM)	15	310 (123.8)	284 (106.8)	317 (122.1)	52 (4.9)
H. armigera					
DMDP	15	323 (78.9)	213 (45.9)	432 (67.9)[a]	32 (6.7)[a]
CYB-3	30	387 (56.9)	287 (134.9)	324 (56.8)	45 (5.9)
DAB-1	50a	386 (137.9)	286 (164.5)	422 (151.6)[a]	40 (2.9)[a]
DNJ	50a	324 (122.9)	253 (143.7)	244 (43.8)	48 (7.9)
DMJ	15	328 (195.9)	277 (186.9)	249 (165.9)	42 (6.9)
Control (NaCl 50 mM)	15	386 (122.9)	276 (85.9)	297 (100.4)	54 (5.8)

[a]Significantly different from the control (Mann–Whitney U test, $P < 0.05$).

TABLE 2. SUMMARY OF BEHAVIORAL EFFECTS IN CHOICE AND NO-CHOICE BIOASSAYS WITH COMPOUNDS AT ALL CONCENTRATIONS TESTED[a]

Bioassay	*S. littoralis*		*S. frugiperda*		*H. virescens*		*H. armigera*	
Compounds:	C	N-C	C	N-C	C	N-C	C	N-C
DMPD	+	+	+	+	+	+	+	+
CYB-3	0	0			0		+	0
DAB-1	+	+	0	0	0		+	+
DNJ	0	0	+	+	0	0	0	0
DMJ	0	0	0	0	−	−	0	0
BR1	0	0	+	0	+		0	
Fagomine	0	−	−	0	0		0	
XZ-1	0	−	−	0	0		0	
Swain	+	+						
Cast	+	+	0	0	+	+	+	
Alexine	0	0	0	0	0		0	

[a]Bioassays: C = choice bioassay, N-C = no-choice bioassay. Nature of behavioral effect: + = significant antifeedant for at least one of the concentrations tested; − = significant phagostimulant for at least one of the concentrations tested; 0 = not significant at any of the concentrations tested. Choice test = Wilcoxen matched paired test, $P < 0.05$, no-choice = Mann–Whitney U test, $P < 0.05$.

D and that to the sugars by neuron C. The activity of these two neurons was considered separately when the sensilla were stimulated by two series of solutions. In one series (Figures 5a–d, points 1–3), the sugar concentration was increased and in the other series (Figure 5a–d, points 3–5), the alkaloid concentration was increased. Each point on the graph is the mean response from five insects, except for point 3, which contains data from both series of solutions and is therefore the mean from 10 insects. We were able to pool the data from each series because they did not differ significantly. Thus, the neural response to stimulus 3 after stimulations with the sugar–alkaloid combinations 1 and 2 was similar to that obtained when stimulus 3 was the initial stimulation of the series 3, 4, and 5. In the interests of clarity, the standard errors (5–15% of the mean response) associated with the means have been omitted from Figures 4a,b, and 5a–d.

In general, the firing rate of neuron C increased as the sugar concentration was increased; then, when the highest sugar concentration was maintained and the concentration of alkaloid increased, the response to neuron C declined as the alkaloid concentration of the stimulating cocktail increased. With increasing

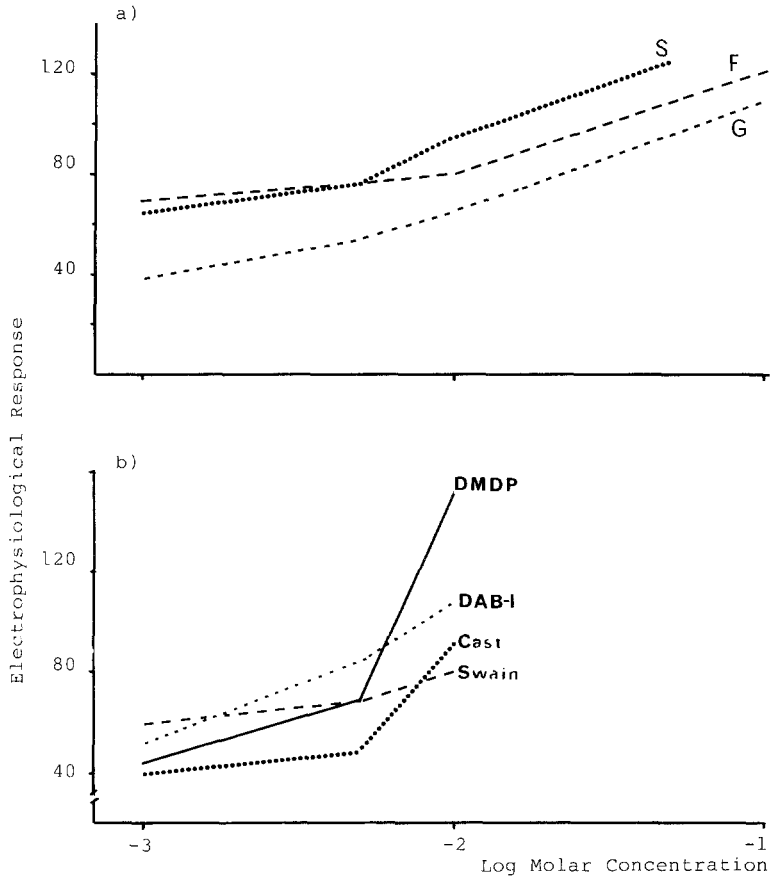


FIG. 4. Electrophysiological responses (total impulses in first second from lateral sensilla) of *S. littoralis* larvae to stimulation with sugars (a) and alkaloids (b) at various concentrations. S = sucrose, F. = fructose, G = glucose, Cast = castanospermine, Swain = swainsonine. (N = 15.)

alkaloid concentration, the response of neuron D increased accordingly. Furthermore, the overall responses to the combination solutions were lower (Figure 5a–d) than to the sugars or alkaloids along (Figure 4). It would appear that one effect of the alkaloid is to reduce the firing of neuron C, the neuron mainly responsive to sugars. The extent to which this occurred varied with different combinations of alkaloid and sugar (Table 3). For example, DMDP reduced the response of neuron C to fructose more than that to sucrose or glucose (Fig-

ure 5a and Table 3). Overall, castanospermine affected the response of neuron C to glucose more than that to sucrose or fructose (Figure 5c and Table 3), whereas DAB-1 affected the response to sucrose more than that to glucose or fructose (Figure 5b and Table 3). Swainsonine, which inhibited the response to sugars least, had its greatest effect on the response of neuron C to low concentrations of fructose [Figure 5d (combinations 1 and 2) and Table 3]. Conversely, swainsonine had the greatest capacity to stimulate neuron D in the presence of the sugars, especially at the higher concentrations used (Figure 5d).

Figure 6a–d shows that a single stimulation with one of the test alkaloids decreases the subsequent response, tested after an interval of 45–63 min, to each of the three sugars. The decrease in response or "inhibition" was greatest with DMDP, DAB-1, and castanospermine. The variation in the duration of this inhibition indicated some specificity in the interactions between the sugars and alkaloids. DMDP still affected the second response to fructose, applied 99 min after the alkaloid stimulation, whereas the second response to sucrose and to glucose was similar to the initial, pre-DMDP treatment, response (Figure 6a). DAB-1 inhibited the second response to sucrose more than that to either fructose or glucose (Figure 6b). Castanospermine affected the second response to glucose and sucrose but not to fructose (Figure 6c), whereas swainsonine affected only the second response to glucose (Figure 6d).

DISCUSSION

Despite the fact that many of the compounds tested here have structural similarities to the common phagostimulatory sugars, some act as antifeedants. In addition, the antifeedant activity of a given compound varies between species, although a few compounds produced very similar effects in all four species. For example, fagomine, XZ-1 and alexine were always ineffective as antifeedants, whereas DMDP was a consistently effective antifeedant.

The antifeedant effect, when it occurred, resulted in a reduction of the amount eaten, either by reducing meal size or by increasing the intervals between meals. Generally, the first meal taken was of greater duration than the second. This decrease in meal size suggests that the antifeedant effect increases with time. This could be due to changes occurring in the sensitivity of the taste receptor system (Blaney and Simmonds, 1987) or to deleterious metabolic effects influencing the feeding behavior of the insect (Dethier, 1980; Bernays and Lee, 1988).

Some polyhydroxy alkaloids did not produce dose-dependent neural or behavioral responses. In these cases there was a high degree of variability between larvae within a species. The alkaloids involved, such as alexine, gen-

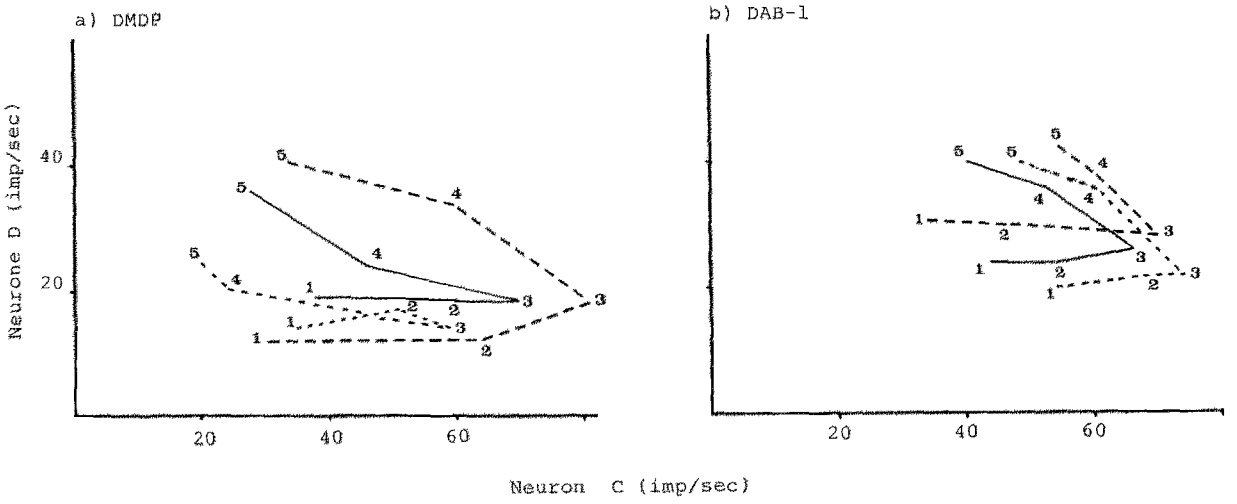


FIG. 5. Electrophysiological responses (impulses in first second) in neurons C and D in the lateral sensilla of larvae of *S. littoralis* to stimulation with the alkaloids DMDP (a), DAB-1 (b), castanospermine (c) and swainsonine (d) in combination with the sugars sucrose (———), fructose (- - - -) and glucose (– – –). Alkaloid concentrations: points 1–3 = 1 mM, point 4 = 5 mM, point 5 = 10 mM. Sugar concentrations: point 1 = 1 mM, point 2 = 10 mM, points 3–5 = 100 mM (fructose and glucose) or 50 mM (sucrose). (N = 10.)

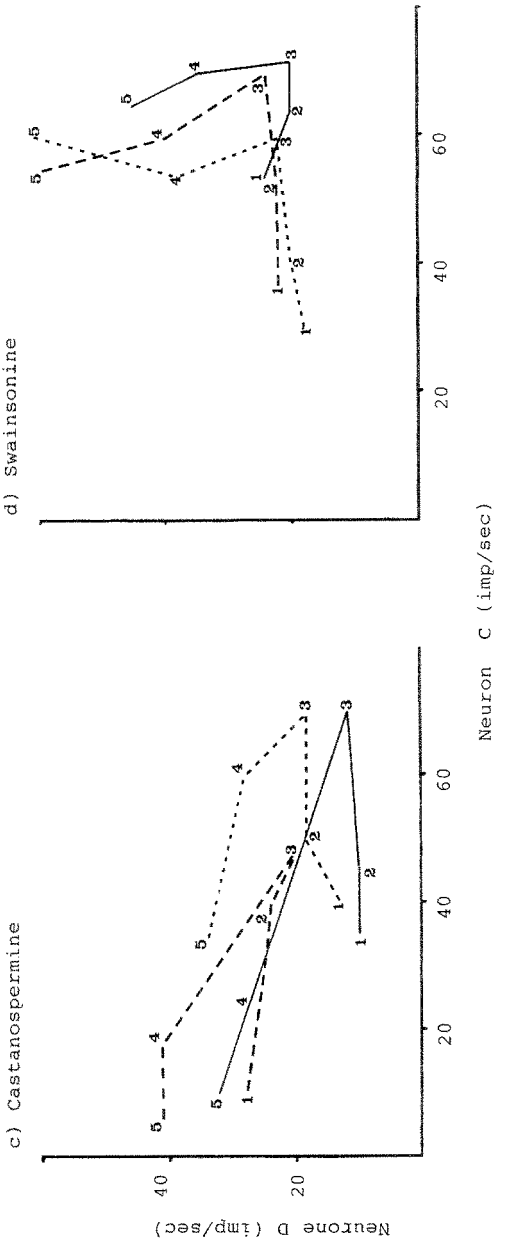


FIG. 5. Continued

TABLE 3. REDUCTION IN FIRING RATE (%) OF NEURON C IN LATERAL SENSILLA OF *S. littoralis* WHEN STIMULATED WITH SUGAR–ALKALOID COMBINATIONS COMPARED TO RESPONSE TO SUGAR ALONE[a]

	Inhibition of sugars (%)														
	Fructose					Glucose					Sucrose				
Alkaloids	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
DMDP	42	32	47	78	82	0	9	26	58	74	42	50	44	63	78
DAB-1	16	14	35	44	55	0	31	41	50	54	36	42	47	58	68
Cast.	32	34	34	36	57	72	40	46	76	76	29	36	36	86	91
Swain.	50	47	36	47	40	0	0	18	47	40	0	24	34	36	41

[a]Concentrations of sugars and alkaloids in solutions 1–5 as in Figure 5.

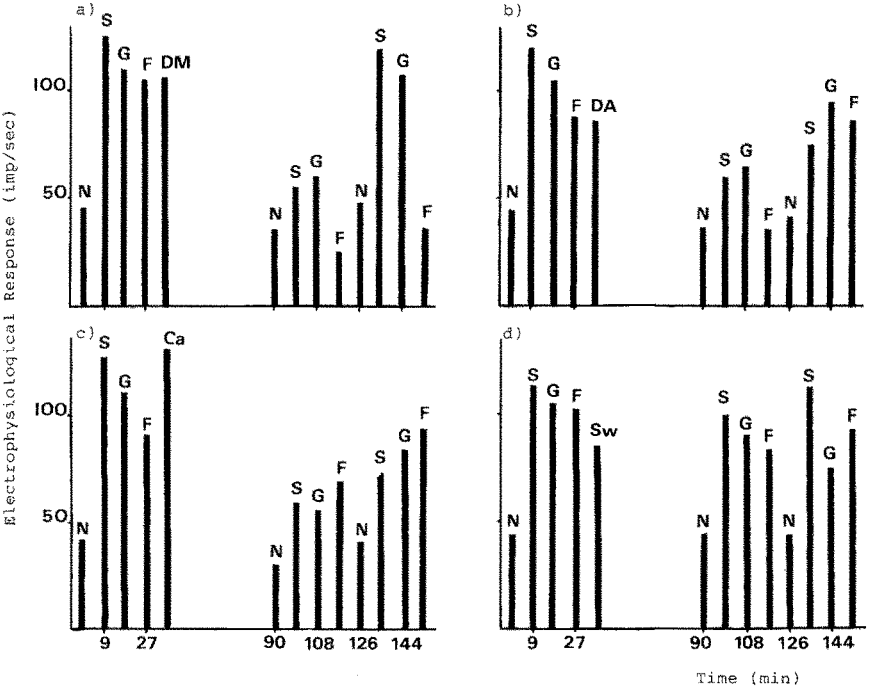


FIG. 6. Electrophysiological responses (total impulses in first second from lateral sensilla) of *S. littoralis* larvae to stimulation with 50 mM NaCl (N), 50 mM sucrose (S), 100 mM glucose (G), 100 mM fructose (F) before and after stimulation with the alkaloids (1 mM) DMDP (DM) (a), DAB-1 (DA) (b), castanospermine (Ca) (c), and swainsonine (Sw) (d). ($N = 15$.)

erally did not stimulate neuron D preferentially in either the lateral or medial styloconic sensilla.

It is interesting that in both *H. virescens* and *H. armigera* the neural and behavioral responses to castanospermine were dose-dependent, but the responses increased as the concentration of castanospermine decreased. We have no explanation for this phenomenon.

Generally, there is an identifiable relationship between the neural activity recorded and the behavioral response associated with it. However, for a given compound, that relationship is not always the same when insects from different species are tested. For example, DMDP produced a similar rate of firing in neuron D and a similar behavioral response in both *S. frugiperda* and *H. virescens*. However, although the behavioral response of *S. littoralis* to DMDP was very similar to that of these two species, the neural input associated with it was considerably less. On the other hand, in *H. armigera*, DMDP produced a much higher neural response in neuron D, and this was associated with a much greater level of antifeedant activity than that shown by any of the other species.

The behavioral responses recorded were less variable than the neural input associated with them. This may reflect the fact that in these experiments we do not have access to the total neural input from all the taste receptors involved in food selection. There are other chemosensory systems associated with the mouthparts that may influence food selection (Schoonhoven, 1987). For example, taste receptors on the maxillary palps are known to have a role in assessing food quality (Ishikawa et al. 1969; Hanson and Dethier, 1973), and the epipharyngeal receptors have been shown to be important in eliciting swallowing and therefore play a part in selection behavior (de Boer et al., 1977). However, by means of ablation experiments, Hanson and de Boer (1987) showed that when only the medial styloconic sensilla containing what they term the "deterrent neuron" remain, *Manduca sexta* was still able to discriminate between potential food materials. Thus, although the neural input that we have used in these experiments is incomplete, it is not inappropriate.

Even if we are recording the most important neural input used in food selection, our measure of it may not be the most appropriate indicator of the information on which the insect bases a behavioral response. Previous studies have shown that the frequency of neural input recorded from a sensillum during the first second of stimulation correlates well with a range of behavioral responses, such as first meal duration (Blaney et al., 1988), time spent feeding on a treatment disk (Blaney et al., 1987) and the amount of fecal material produced (Ma, 1972; Blom, 1978). The possibility remains, however, that the insect uses some other code for taste quality, such as assessment of the temporal sequence of firing, which gives a continuous evaluation of the activity of individual neurons. It is also likely that simultaneous evaluation of inputs from different neurons allows contradictory signals, indicating phagostimulants or

antifeedants, to be assessed concurrently (Schoonhoven, 1987). In the present study, there was less variability and better correlation with antifeedant behavior when the output from neuron D in the lateral sensillum was taken to represent the neural information than when the output from all the neurons was taken together. Thus, the total firing rate from a sensillum is an imperfect measure of the sensory input on which food selection is based. However, when, as in this study, the response of neurons signaling phagostimulation and others signaling antifeedants can be distinguished, a closer approximation to the information available to the insect is obtained.

In the present case, neurons C and D signal phagostimulation and antifeedant activity, respectively. The antifeedant activity of a compound may be associated with a high rate of firing in an antifeedant neuron or, when the test compound is combined with a sugar, with reduced firing in both antifeedant and phagostimulant neurons at the same time. Those alkaloids that induced the highest firing rates in neuron D in the absence of sugars also showed the greatest antifeedant effect in the choice bioassay. However, in nature the insects will seldom encounter compounds in isolation, and consequently the neural responses to mixtures of compounds are of greater interest.

In this context, a notable feature of our experiments is that the responses of identifiable neurons to specific compounds, in this case alkaloids or sugars, are greater in magnitude when the compounds are applied singly than when an alkaloid and a sugar are applied together. This phenomenon has been described by us before and designated "interaction" (Simmonds and Blaney, 1984, 1990). Our previous work investigated, in particular, the interaction between azadirachtin and sucrose. These compounds stimulate different neurons in the medial sensillum of *S. littoralis*. It was found that increasing the concentration of either compound in the stimulus mixture resulted in a significant decrease in the firing rate of both neurons (Simmonds and Blaney, 1984). In the present work, the interaction when alkaloid and sugar were presented together was less extreme. In general, increasing the sugar concentration did not decrease the firing rate of neuron D, but increasing the alkaloid concentration did decrease the firing rate of neuron C, except in the case of swainsonine where the latter effect was not significant.

The polyhydroxy alkaloids are well known for their ability to inhibit the action of glucosidases, and this may have some bearing on their effect on the insect taste system (Fellows et al., 1989). The sugar receptors of flies have been investigated with a view to determining the nature of receptor sites for sugars on the dendritic membranes (Nakashima et al., 1982; Ozaki, 1988; Shimada, 1978; Wieczorek and Koppl, 1982; Wieczorek and Wolff, 1989; Wieczorek et al., 1988). Several studies have identified at least two types of receptor site, called pyranose and furanose sites, to account for their apparent affinity for pyranose and furanose sugars (Shimada et al., 1974; Wieczorek et al., 1988).

One line of investigation has suggested the involvement of α-glucosidase in the receptor mechanism, and it has been suggested that the enzyme may act as a receptor protein (Hansen, 1974; Amakawa et al., 1975). If this were so, we could expect some of the alkaloids used in this study, which are known inhibitors of glycosidases, to shed some light on the mechanism involved. For example, Nakashima et al. (1982) have shown that the alkaloid nojirimycin, a known glucosidase inhibitor, competitively inhibits the membrane-bound α-glucosidase in the labellar sensilla of *Phormia regina*.

Although the antimetabolic activity of DMDP and castanospermine has been attributed to their ability to inhibit glucosidases in insect guts (Evans et al., 1985; Fellows et al., 1989), there is no direct evidence that their antifeedant activity is a result of the inhibition of glucosidases in the insects' taste sensilla. The results show that DMDP did affect the response to the pyranose sugars, and this might be attributed to it inhibiting the α-glucosidase thought to be associated with the pyranose site. However, its major inhibiting effect was seen with the furanose sugar fructose. One possible explanation is that the furanose site is also an α-glucosidase, but there is no evidence in the literature to support this. This leaves the possibility that the effect of DMDP may depend on its structural form and its resemblance to fructose. This may allow it to occupy the furanose sites and, in doing so, may have blocked these sites; this could result in the reduced response to fructose seen here, which persisted up to 90 min after stimulation with DMDP. It should be noted that when DMDP is applied to the sensillum in the absence of a sugar, it stimulates neuron D preferentially, with neuron C responding only very slightly. It may be that the furanose and pyranose sites are blocked in this situation, but we have no means of knowing it: it is only when a sugar is applied and the expected response from neuron C does not occur that the blocking becomes apparent. With DMDP this blocking affects the furanose sites much more than the pyranose sites. With castanospermine, DAB-1, and swainsonine, it is the pyranose site rather than the furanose site that appears to be preferentially blocked. The results with swainsonine are equivocal because, in the sequential experiment just discussed, it is the response to glucose that is affected, whereas when combined with a sugar, it appears to affect the response to fructose.

The interpretations offered here on the role of the alkaloids as inhibitors of pyranose and furanose receptor sites are based on earlier work carried out exclusively on flies. The situation in caterpillars may be different. Indeed, there appear to be differences between species of flies. Recently, Wieczorek and Wolff (1989) have shown that the pyranose site in *Drosophila melanogaster* has a more rigid stereospecificity than in the calypterate flies. They also showed that in *D. melanogaster* D-fructose, which stimulates the furanose site in calypterate flies (Shimada et al., 1974; Hanamori et al., 1974), binds to a pyranose site and not to a furanose site. The polyhydroxy alkaloids offer a means to investigate fur-

ther the role of glucosidases in taste systems and are useful tools in our investigation of the molecular basis of the chemospecificity of taste neurons.

Despite the reservations expressed by Jermy (1990) about the usefulness of electrophysiological techniques in the evaluation of compounds for antifeedant activity, we feel that when such techniques are used in combination with detailed behavioral observations, significant advances can be made. Electrophysiology allows the prescreening of minute quantities of newly isolated or synthesized materials; in addition, as shown here, the technique helps in the elucidation of the mechanisms underlying sensory perception. Structure–activity studies can be extended by the use of electrophysiology to give information on the molecular processes underlying host-plant choice and may identify groups of compounds that have particularly useful modes of action.

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STIMULO-DETERRENT DIVERSION: A CONCEPT AND ITS POSSIBLE APPLICATION TO ONION MAGGOT CONTROL

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Abstract—Considerable basic information has been gathered on the interaction between the onion fly (*Delia antiqua*) and its host plant, the onion (*Allium cepa*). An attempt is underway to manipulate ovipositional behavior of this pest by treating onion seedlings with chemical deterrents while simultaneously providing deeply planted onion culls on which onion flies prefer to lay. This bipolar strategy of behavioral manipulation, termed "stimulo-deterrent diversion" (SDD), has the advantages of: (1) avoiding severe pest deprival and concomitant overriding of deterrents, (2) combining the effects of "push" and "pull" multiplicatively, and (3) providing opportunities for enhanced biological control in sites where the pest becomes concentrated. The suggestion is made that using SDD along with soil insecticide might relax or even reverse selection for physiological resistance of *D. antiqua* to insecticides. As tools of molecular biology open new possibilities for manipulating plants and their allelochemicals, applied chemical ecologists should consider arranging situations where the allelochemicals have clear and adaptive messages for the pest. By combining toxins and deterrents at sites where feeding should be prevented, while simultaneously expediting use of alternative plants or plant parts, it might be possible to guide pest evolution toward paths of less conflict with human interest.

Key Words—Oviposition, deterrents, trap crop, behavioral manipulation, push–pull strategy, insecticide resistance management, onion fly, *Delia antiqua*, Diptera, Anthomyiidae.

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INTRODUCTION

Insecticides are a highly convenient and potent means of short-term insect control for most crops, including "minor" but unusually valuable crops like vegetables. However, options for insecticidal control are dwindling due to: (1) increasing demands for residue-free foods, (2) increasing physiological resistance of pests to previously effective chemicals, and (3) decreasing interest by the agrochemical industry in servicing these "lower profit/higher risk" markets. Thus, pest managers must search for supplements or, in some cases, replacements for conventional pesticides.

Cultural controls, such as proper crop rotation and spatial designs favoring sustained biological control ultimately may be the best solutions. However, such practices are currently unpalatable to large-scale vegetable producers who gain a competitive advantage by specializing on two or three select crops. Specialized equipment then can be employed quickly and reliably as dictated by weather and markets. While capital investments are high, labor costs are reduced. Very importantly, large-scale producers who reliably deliver produce of high quality enjoy premium markets not available to the intermittent producer.

We have been exploring how attractants/stimulants and repellents/deterrents might supplement conventional insecticidal control of the onion fly, *Delia antiqua* (Meigen). The goals of this paper are to: (1) describe a control strategy that simultaneously uses stimulants and deterrents, and (2) present results and views on how this strategy might be applied to *D. antiqua*.

FRAMEWORK FOR VIEWING INSECT CONTROL STRATEGIES

Parameters of Damage

Crop damage from insects can be approximated by the expression:

$$\text{Damage} \propto D \cdot A \cdot S \cdot T \qquad (1)$$

where D is pest density in a unit of crop; A is plant acceptability (sensu Singer 1986) to the insect; S is plant suitability to the insect; and T is time of interaction. Some insect control strategies reduce nearly all of these parameters while others affect only one or two (Table 1). Because they kill pests, applied insecticides dramatically reduce D, S, and T. Lack of effect upon A can be an advantage, as willingness of a pest to feed increases lethality.

Resistance of some crop cultivars can be explained by antixenosis (reduced A) (Harris et al., 1987b); however, antibiosis (reduced S) is the trait most sought by plant breeders (Maxwell and Jennings, 1980). Like insecticides, plant-pro-

TABLE 1. ASPECTS OF DAMAGE GENESIS INDUCED BY KNIPLING'S (1979) CATEGORIES OF INSECT CONTROL

Control method	Impact			
	Insect Density	Plant Acceptability	Plant Suitability	Interaction Time
Chemical insecticide	↓↓↓↓[a] kills		↓↓↓↓ renders plants acutely toxic	↓↓↓↓ most kill quickly
Resistant Varieties				
Antibiosis	↓↓↓		↓↓↓	↓↓↓
Antixenosis	↓ reduces finding	↓↓		
Applied deterrents/ antifeedants	↓ reduces finding	↓↓ precludes sustained feeding or oviposition	↓ some antifeedants are somewhat toxic	
Sterilants/genetic manipulation	↓↓↓ reduces reproduction			
Cultural and sanitary control	↓↓↓ increases mortality reduces reproduction reduces finding	↓ e.g., planting date	↓	↓ e.g., early harvest
Biological control	↓↓↓ increases mortality			↓↓
Integrated control	↓↓↓	↓↓	↓↓↓	↓↓

[a] Number of arrows proportional to magnitude of the effect.

duced antibiotics can stand alone as convenient controls, so long as the pest remains physiologically susceptible.

For insect herbivores, we suggest that behavioral modifiers, used singly and as conventional insecticides, will rarely be effective; they do not sufficiently impact D, A, S, and T, or combinations thereof. The thesis of this paper is that behavioral modifiers will be best used in combination with one another and/or with other control strategies.

Dynamic Acceptance Thresholds

Interaction between an insect and a deterrent is likely to be more extended than that between an insect and an insecticide. The usual outcome for an insect encountering an insecticide is rapid death. By contrast, an insect encountering a plant releasing a feeding or ovipositional deterrent is likely to move to plants nearby. If these also contain deterrents, the insect becomes increasingly hungry or gravid. As suggested by the rolling-fulcrum model of host acceptance (Dethier, 1982; Miller and Strickler, 1984), the outcome from exposure to a given set of external excitatory and external inhibitory inputs changes with deprival. The effect of a given level of deterrent would diminish as increasing internal excitatory inputs lower acceptance thresholds over time. Loss of control is likely to be exacerbated if the pest's sensory system accommodates to the deterrent or if learning (Papaj and Prokopy, 1989) reinforces breaches in deterrency.

STIMULO-DETERRENT DIVERSION

The rolling-fulcrum model predicts that deterrents might not be overcome if pests experiencing mounting deprival could intermittently feed or oviposit on a highly acceptable resource. In addition to reducing A of equation 1 by extending deterrent effectiveness, a highly stimulatory alternative resource could also reduce D and T by diverting the pest from the crop. Using the "trap crop" approach simultaneously with deterrents in/on the valued resource yields a bipolar strategy of behavioral manipulation we now call *stimulo-deterrent diversion* (SDD). "Diversion" is now emphasized over trap cropping (Miller, 1986) because: (1) the method should be inclusive of nonherbivores as well as plant pests, and (2) trap cropping has come to connote that no treatment is applied to the valued resource (Evans, 1984). "Stimulo" and "deterrent" are used here in the broad sense of promoting and depressing damaging activities such as feeding and/or ovipositing. As this paper was being revised, we became aware of the not yet widely known reports of M. Rice and Australian colleagues who have been developing this control concept independently using the name push–pull strategy (Rice, 1986; Pyke et al., 1987). Although other researchers may

have made reference to using combinations of stimulants and deterrents for pest control, this paper and those by Rice are the first to formalize the concept.

The advantages of SDD could be greater than initially expected. Key considerations are the potential interactions between forces of "push" and "pull," and how pest control might be sustained indefinitely.

BACKGROUND ON ONION (*Allium cepa L.*) (LILALES: AMARYLLIDACEAE)

A. cepa is a biennial (Jones and Mann, 1963) usually planted as seeds by commercial growers. The most notable feature of onion is distinctive chemistry valued for flavoring. *A. cepa* and other alliums contain unusually high levels of organic sulfur in the form of alkylcysteine sulfoxides and γ-glutamyl peptides, derived form cysteine and glutamate, respectively (Whitaker, 1976). When *Allium* cells are disrupted, previously compartmentalized S-containing substrates react with alliinase to liberate a family of simple alkyl sulfides whose composition is determined by the proportions of S-methyl-, S-1-propenyl-, and S-2-propenyl-cysteine sulfoxides present as precursors (Freeman and Whenham, 1976). The major products from *A. cepa* are *n*-dipropyl disulfide $(CH_3CH_2CH_2{-}S{-})_2$ and propanethiol $(CH_3CH_2CH_2{-}SH)$ (Block, 1985, et ante).

Most of the onions produced in temperate North America are planted on organic (muck) soils reclaimed by draining late-successional lake bottoms. The calendar of events outlined in Figure 1 is typical for Michigan onion culture. Because of high investments in specialized equipment, many Michigan growers dedicate much of their acreage to continuous onion production. If they rotate the crop, distances to new fields are usually inconsequential.

BACKGROUND ON ONION FLY [*Delia antiqua* (Meigen)] (DIPTERA; ANTHOMYIIDAE)

D. antiqua specializes on onion and a few other propyl sulfide-producing alliums (Ellis and Eckenrode, 1979). Onion maggot is the most serious pest of onions throughout temperate portions of the Northern Hemisphere (Loosjes, 1976). Densities of overwintering *D. antiqua* pupae in typical Michigan fields under continuous onion culture were estimated about 10,000–30,000/ha (Drummond, 1982). This compares well with Loosjes' (1976) estimate of typical pupal densities in The Netherlands. When control was not maintained, *D. antiqua* populations surpassed 500,000/ha (Finch and Eckenrode, 1985).

For Michigan, the first of three onion fly generations emerges in May and early June (Figure 1), when the onions are seedlings. After sufficient feeding to mature eggs, females oviposit in the soil adjacent to onion plants. Under field

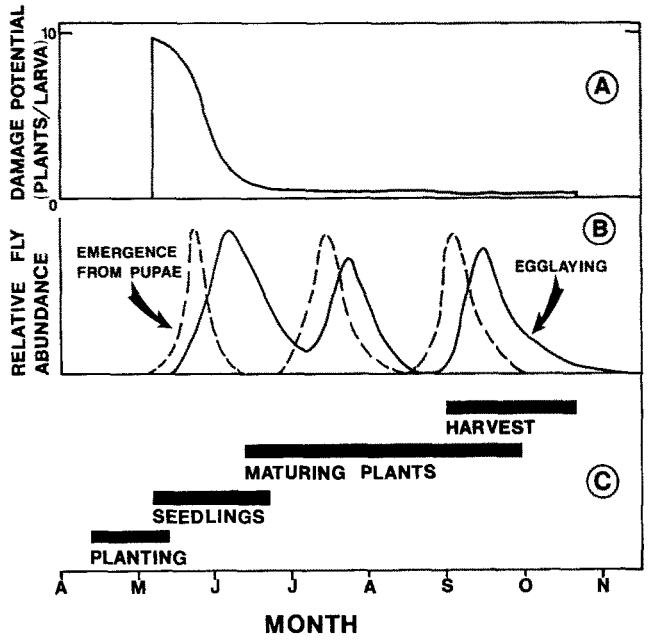


FIG. 1. Comparative phenologies of onions and the onion fly in Grant, Michigan. As indicated in (A), one larva can damage many plants in the early spring. The ovipositional curve was estimated by fly response to traps baited with enzymatic yeast hydrolysate.

conditions, estimated lifetime egg production was ca. 50 per female for spring flies and even fewer for the second and third generation of flies (Loosjes, 1976). Presumably, fecundity in the field is strongly influenced by adult diet, as females fed artificial diets (Schneider et al., 1983) under optimal laboratory conditions produce several hundred eggs (Vernon and Borden, 1979; Weston, 1986).

Larval *D. antiqua* destroy plants by feeding on subterranean tissues and by facilitating microbial infections. One larva can destroy as many as 10 plants in the one-to-two-leaf stage before pupating (Workman, 1958). As plant size increases, damage potential of each maggot decreases (Figure 1), both because each plant reaches a size that can support development of tens of larvae and because onion flies tend to concentrate eggs upon previously infested onion plants (Workman, 1958; Dindonis and Miller, 1980a). Thus, emphasis on control is directed at the spring generation of *D. antiqua*.

In North America, onion maggot is currently controlled by applying the organophosphate insecticides chlorpyrifos or fonofos in the row at seeding. However, this pest is notorious for developing physiological resistance to insecticides (e.g., Harris et al., 1982). As current insecticidal control is temporary,

alternative or supplemental control strategies are needed, including behavioral manipulation.

D. antiqua OVIPOSITIONAL BEHAVIORS

Host-plant finding by onion flies can involve orientation to onion odors (Dindonis and Miller, 1980b; Miller et al., 1984; Judd and Borden, 1988) as well as visual cues (Harris and Miller, 1983). Given the high likelihood of fly emergence near onions in current agronomic situations, it is also likely that many encounters between insect and host plant are incidental. The data of Martinson et al. (1988; 1989) suggest appreciable random movement.

Onion fly oviposition is stimulated by the complex of visual-physical and chemical cues mirroring those from an onion plant at the three-to-four-leaf stage (Harris and Miller, 1988, et ante). Important features of this "Gestalt" are a green or yellow cylinder (foliage) 4–8 mm in diameter, at least 10 cm long, and standing vertically in soil. Oviposition is facilitated by Pr_2S_2 at appropriate concentrations (Harris et al., 1987a) and is stimulated even more strongly by volatiles emitted by soft-rotting bacteria colonizing onions (Hausmann and Miller, 1989). Healthy young onion plants can be mimicked by painted glass tubes coated with Pr_2S_2-containing (0.05%) paraffin wax (Harris et al., 1987a). Additionally, oviposition is facilitated by soil crevices *ca.* 1 mm wide and deeper than the 4-mm-long *D. antiqua* ovipositor (Mowry et al., 1989a).

Before depositing eggs, onion flies examine a potential host using vision, mechanoreception, olfaction, and taste while sitting on and/or running over the plant. Examining behaviors are highly probabilistic; moreover, specific sensory cues affect various rather than particular behavioral transitions (Harris and Miller, 1991).

By contrast, egg deposition is highly deterministic (Mowry et al., 1989b) and appears to be governed by a motor program behaving as a fixed action pattern. Egg depositional bouts produce clutches of about two-to-four eggs and are interspersed with examining bouts. Ovipositional behaviors are virtually indistinguishable on artificial vs. real plants (Harris and Miller, 1991). The highly reproducible and manipulable surrogate onions have become an important tool for research on ovipositional deterrents (Cowles et al., 1988, 1990) as well as internal physiological factors modulating responses to host-plant cues.

Some ovipositional specialists such as certain *Dacus* fruit flies (Fitt, 1986) and the cabbage fly, *D. radicum* (Nair and McEwen, 1976), cease maturing eggs and will not oviposit in the absence of host plants. By contrast, in the laboratory, *D. antiqua*, does not slow oogenesis, vitellogenesis, or deposition, even in the complete absence of host plants (Weston, 1986); females will lay the full complement of eggs in cracks and crevices in their cages (e.g., under the water dish) if onions are not provided in ovipositional cups. In terms of the

rolling-fulcrum model of resource acceptance (Miller and Strickler, 1984), *D. antiqua* has a decision fulcrum that moves far to the right upon deprival of normal host-plant stimuli. Although deprived onion flies appear to maintain the normal rank order for acceptance of hosts, their host range broadens decidedly. Such a trait bodes poorly for pest control by conventional behavioral modification or at least suggests that the control strategy must be designed to overcome this phenomenon.

ATTEMPTS TO CONTROL ONION MAGGOT BY TRAP-CROPPING

To determine what host material was most stimulatory for *D. antiqua* oviposition and whether such material would protect insecticide-free onion seedlings nearby, these treatments were deployed in 2.3 × 2.3-m plots in a linearly arranged randomized complete block design replicated six times: (1) large onion bulbs planted so the neck was buried 3–4 cm below the soil surface, (2) onion sets planted as for (1), and (3) normally seeded onions as a control. Spacing between plots and blocks was 50 m. One complete row running through the middle of each plot and the length of the field was left untreated with soil insecticide so that stand loss could be assessed both inside and outside of the plots. The hypothesis was that larger onion plants would receive the most eggs both because of their more stimulatory size (Harris and Miller, 1984) and because microorganisms colonizing sprouted onion bulbs release volatile attractants (Miller et al., 1984) and ovipositional stimulants (Hausmann and Miller, 1989).

D. antiqua eggs were extracted from soil samples (60 cm of row) taken during the first generation of fly activity. As suggested by the early work of Lovett (1923) and Davis (1932), deeply planted large bulbs were by far the best egg sinks (Figure 2). Onions grown from sets received far fewer eggs then did

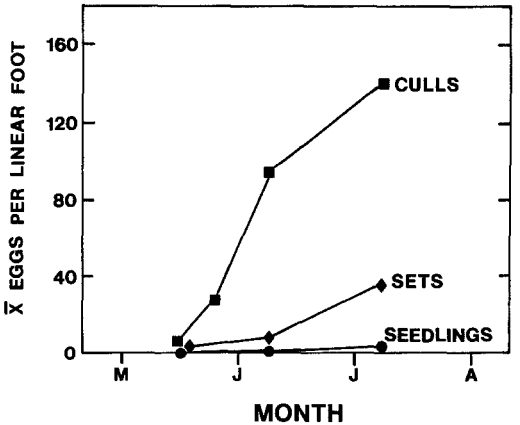


FIG. 2. Densities of onion fly eggs accumulating on three different types of onion plants deployed in a large commercial onion field in Eaton Rapids, Michigan, during 1984.

large onion culls, but more than plants grown from seeds. Early in the season, no eggs were found on seedlings; however, by July several eggs were found per sample. Damage in untreated seedling rows was about 10% and did not improve in close proximity to the plots of culls. We have established (Cowles, 1990) that the optimal planting depth of onion culls is with the neck 5 cm below the soil surface and that larval colonization does not limit effectiveness as an egg sink during the first fly generation. Contrary to some enthusiastic reports on using onion culls for trap-cropping (Lovett, 1923; Davis, 1932), we believe this control strategy would, by itself, be unreliable unless fly densities were unusually low.

ATTEMPTS TO CONTROL ONION MAGGOT BY CHEMICAL DETERRENTS OF OVIPOSITION

We have evaluated various herbal preparations (Cowles et al., 1989) and chemical synthetics (Cowles et al., 1990) as *D. antiqua* ovipositional deterrents. Chemicals were applied directly to 4-cm-diameter sand-filled ovipositional cups holding onion surrogates or by diluting synthetics with a polyethylene glycol keeper. Diverse materials are deterrent if applied in high enough dosage. In general, moderately oxygenated but uncharged hydrocarbons in the molecular weight range of ca. 100–200 deter *D. antiqua* oviposition by ca. 90% in choice tests if applied at several hundred micrograms per plant. Several terpenoid preparations, such as pine and cedar leaf oil, previously reported to be deterrents of *D. antiqua* oviposition (Alfaro et al., 1981; Javer et al., 1987), also appear to require high dosages.

The most active materials we have found to date are derivatives of cinnamaldehyde (Cowles et al., 1990); but even these require an atmospheric concentration of 1–2 ng/ml air as determined by GLC of air samples taken around release sites giving 90–95% deterrency in laboratory bioassay (Cowles et al., 1990).

The requirement for high dosages of some of these ovipositional deterrents (e.g., cinnamaldehyde and phenethanol) may be partially offset by their low costs and the likely ease in obtaining registrations for use in pest control. Various of these materials are on the Generally Recognized as Safe (GRAS) list of the United States Food and Drug Administration and are widely used in human foods and perfumes.

We have had limited success protecting onion seedlings by applying macroencapsulated formulations (Kynar beads, charcoal pellets, and plastic tubes) of cinnamaldehyde and phenethanol. Formulation needs more work; the challenge is to keep adequate concentrations of deterrents in place for the duration of the first fly generation, particularly when winds are high and rains heavy. Care must be taken also that fragile seedlings are not burned by direct contact with high concentrations of AI. Very importantly, chemical deterrents alone

tend to lose effectiveness against *D. antiqua* if females are provided no alternative sites upon which to oviposit.

PROGRESS ON DEVELOPING SDD FOR ONION MAGGOT CONTROL

Central questions in contemplating SDD for *D. antiqua* are: (1) what ovipositional preference ratio between diversionary crop (DC) and valued crop (VC) would be needed for stand loss of below 3–5%, and (2) how would this ratio vary with pest density? These relationships are estimated in Figure 3, based on knowledge and some assumptions about the onion/onion fly system: (1) About 4% of the available field area can realistically be used for deploying diversionary crop. (2) There are enough cull onions available in Michigan for their use as diversionary crop; every year ca. 5–10% of the crop is dumped due to bulb sprouting in storage or overproduction. In fact, disposal of onion culls is becoming an environmental problem. (3) The possible threat of introducing plant pathogens from culls could be offset by treating them with fungicides or physically removing them after spring egg laying. (4) Onion flies are mobile enough to interact freely with diversionary and valued crops, so that preference differentials will be expressed fully. Trapping studies of Loosjes (1976) and others have shown diel fly movement into and out of fields. Our unpublished observations of individual flies confirm appreciable movement within fields during any period of the day. (5) Onion flies produce ca. 100 viable eggs (liberal estimate) in the field over a lifetime. (6) Very high numbers of eggs will be laid on individual onion culls. Laboratory and field experiments revealed no

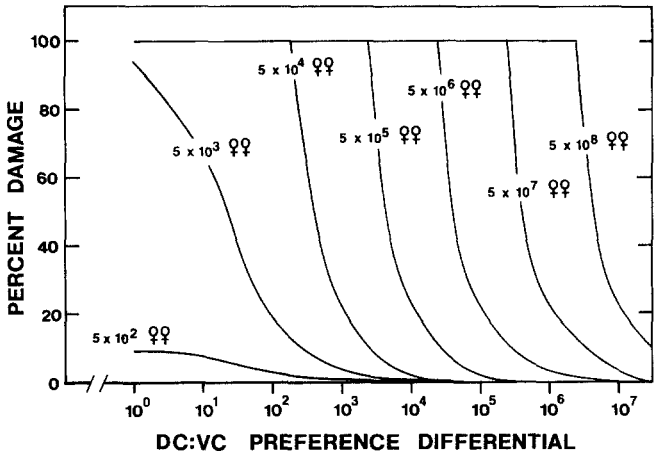


FIG. 3. Percent control anticipated as influenced by diversionary crop (DC)–valued crop (VC) preference differential of onion fly females. Assumptions given in text.

evidence that ovipositional deterrent pheromones are laid down with eggs. (7) Each maggot will kill three plants on average as it develops. (8) SDD will be done with row spacings and plant densities currently employed in Michigan: 400 rows per 100 m and 40 plants per m, respectively. (9) Onion flies will not converge significantly upon the test field. Calculations based on these assertions suggest (Figure 3) that control by SDD or trap-cropping would be highly influenced by pest density. At *D. antiqua* densities typical for Michigan onion fields (10,000–30,000/ha), stand losses of 3–5% would require a DC/VC preference differential of ca. 500:1. Evidence from laboratory and field studies suggests this preference differential is attainable, particularly when seedlings are tiny and treated with deterrent.

SDD of onion fly has been tried in the greenhouse to qualify possible interaction between cull onions and deterrent reducing *D. antiqua* oviposition on seedling onions. The setting was a 1.5 × 1.5 × 3.5-m screened cage (held in a glasshouse) containing 100 mature *D. antiqua* females and 20 males. This fly density was at least 20-fold higher than that in a typical Michigan field. On successive days and according to a Latin-square design, four pots of 3-to-5-leaf onion seedlings were presented to the flies for two days with and without 25 mg cinnamaldehyde adsorbed into 25 mg of <2-mm-diameter charcoal granules and in the presence or absence of a central pot containing two sprouted cull onions. Larger seedlings, a dosage of cinnamaldehyde that was not completely deterrent, and high fly density were used so that some counts were recorded in each cell of the factorial design.

The greatest reduction in oviposition on seedlings occurred when both culls and deterrent were present (Table 2). When not computing from marginal probabilities, 0.04 and 0.48 times the normal numbers of eggs (3185) were laid on onion seedlings, respectively, in the presence of deterrent or culls administered

TABLE 2. NUMBERS OF ONION FLY EGGS ON POTTED ONION SEEDLINGS AS AFFECTED BY PRESENCE OF DETERRENT AND/OR NEARBY CULL ONIONS ACTING AS AN EGG SINK

Treatment	Total eggs	
	No cinnamaldehyde	Cinnamaldehyde
Eggs on seedlings		
Culls absent	3185	127
	(3180)[a]	(132)
Culls present	1531	69[b]
	(1536)	(64)
Eggs on Culls	3867	4211

[a] Numbers in parentheses are expected values computed from marginal probabilities.
[b] $\chi^2_{(1\,df)} = 0.65$ for interaction of main effects ($P > 0.25$).

separately. The 69 eggs actually laid when push and pull were combined compare very well to the 61 predicted from a multiplicative model, (0.04 × 0.48)(3185). When expected values were based on marginal probabilities as in Table 2, the match with those observed was remarkably consistent with a simple multiplicative model. The data of Rice (1986) and Pyke et al. (1987), on reductions in *Heliothis* eggs in cotton plots treated with azadirachtin and in proximity to pigeon pea or maize trap crops. are generally consistent with a simple multiplicative mode. SDD would be justified even more if the combined effect were more than multiplicative.

Even though this experiment (Table 2) was not designed to maximize the effects of presence of culls and deterrent, the DC/VC preference ratio on a per plant basis was 490:1; this gets very close to the 500:1 ratio that Figure 3 predicts would be required for the Michigan situation. Field tests of SDD on *D antiqua* are pending development of deterrent formulations that will reliably dispense deterrent for extended periods during the spring fly generation.

An alternative means of administering deterrents among onion seedlings is companion plantings. This idea is appealing, particularly since many plants synthesize, store, and release quite high levels of volatiles fitting the structural requirements for *D. antiqua* ovipositional deterrents. An advantage of this approach is that the complications of registering synthetic chemicals for pest control might be side-stepped. Traits for an ideal companion plant for onion would be that: (1) seeds would be inexpensive and could be conveniently planted along with the onion seed, (2) it would not compete heavily with the onion seedlings for light and nutrients, (3) it would not be allelopathic, and (4) after the first generation of onion fly activity, it could be removed by a selective herbicide. Michigan onion growers already broadcast barley at two to three bushels per acre over newly planted onion fields to guard against wind erosion. Barley is removed by fluazifop-butyl when ca. 10 cm tall, because a cover crop is no longer needed to hold down the soil. Since this procedure was invented by onion growers, they would be likely to adopt companion plants serving multiple functions.

Unfortunately, our search for aromatic grasses as companion plants for onion has produced no good leads. Of a series of herbs tried as companion plants, carrot, hyssop, and catnip deter oviposition on onion seedlings by 90% in laboratory choice tests; however, no herbicide is yet available for their selective removal.

INTEGRATION OF SDD WITH OTHER CONTROLS

Should behavioral controls be substituted for, alternated with, or combined with insecticidal controls? The disadvantage of added costs and complexity in considering superimposing SDD on insecticidal control must be weighed against

the pronounced advantage that strong selection pressure would be established favoring flies most obedient to the push and pull commands of SDD. Throughout this work we have envisioned that the numbers of flies emerging from the diversionary crop would be regulated so as to preclude pest epidemics. However, it would be important to manage survival of individuals developing in the diversionary crop to be superior to that in the valued crop. Although the idea of looking after the welfare of insect pests is radical, it opens some exciting possibilities for guiding pest evolution so the pest's preference for plants would progressively diverge from the valued crop. For *D. antiqua* under SDD plus insecticide, a situation is envisioned where physiological resistance to insecticides would diminish in a population, while avoidance of deterrent-treated valued crop would increase. Such a population would be easily controlled. Most individuals would develop in the diversionary crop, while those attempting to develop in the valued crop would suffer high mortality. A population genetics model incorporating two-allele loci for avoidance and physiological resistance traits (Cowles, 1990) suggests the most important determinants for selecting an avoiding and physiologically susceptible population are: (1) higher suitability of the diversionary crop, (2) high finding of the diversionary crop, and (3) use of deterrents to which the pest is preadapted for response. This triad of management components (diversionary or refuge crop, deterrent/antixenosis for the valued crop, and insecticide/antibiosis for the valued crop) offers the prospect of sustained control and an exit off the treadmill of needing a new insecticide for the onion maggot approximately every 8–10 years.

An additional possibility deserving attention in the onion fly system is incorporation of natural enemies whose populations could be favored by having the pest concentrated in the diversionary crop. For example, these conditions would favor the pathogenic fungus *Entomophthora muscae* (Carruthers *et al.*, 1985) as well as insectan natural enemies of *D. antiqua*, e.g., the carabid predator *Bembidion quadrimaculatum* (Grafius and Warner, 1989), and the parasitoids *Aleochara bilineata* and *Aphaereta minuta* (Loosjes, 1976; Groden, 1982).

POSSIBLE FUTURE DEVELOPMENTS

It is hoped that the principles emerging from this work on onion fly transcend this given system. Perhaps SDD will prove easier to perfect for other systems. Some questions needing attention are: (1) Do the components of SDD always act multiplicatively? (2) Is SDD more promising for pests less affected by host deprival? (3) What is the effect of pest mobility and degree of host specialization? SDD may work best for highly mobile generalists that move further from a treated area. (4) Can insecticide resistance actually be reversed by using combinations of toxins, deterrents, and refuges?

As genetic engineering of plants develops, serious attention should be

directed to the designing of ideal companion plants. It will be better both energetically and politically to have plants produce desired allelochemicals at the site where they are needed than to synthesize, formulate, and spray chemical synthetics. Commercial interests might need to continue to shift from chemicals to seeds.

SDD might be applied on the scale of an individual plant, e.g., directing pests away from higher- to lower-valued plant parts. As discussed above for the field-scale situation, plant engineers would need to provide evolutionary incentive for the pest to diverge from using the plant parts that are the human objective of crop production. As they have done over insect–plant evolutionary time, plant allelochemicals will continue to play an important role in guiding insect host-plant use. As molecular biology opens new possibilities for manipulating plants and their allelochemicals, applied chemical ecologists should arrange situations where the allelochemicals have clear and adaptive messages for the pest. This approach could lead to greater sensitivities and responsiveness of pests to allelochemicals, more humanly desired outcomes of insect/plant interactions, and it is hoped, sustained rather than temporary pest control.

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CUTICULAR HYDROCARBONS AND DEFENSIVE COMPOUNDS OF *Reticulitermes flavipes* (Kollar) AND *R. santonensis* (Feytaud): POLYMORPHISM AND CHEMOTAXONOMY

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Abstract—Colonies of *Reticulitermes flavipes* and *R. santonensis* were collected from the southeastern United States (Georgia) and the southwest of France (Charente-maritime). Defensive compounds and cuticular hydrocarbons were identified by gas chromatography–mass spectrometry and quantified by gas chromatography using an internal standard for each caste and all colonies. These analyses show that although the cuticular hydrocarbons of *R. santonensis* in Europe and *R. flavipes* in Georgia are identical, their relative proportions are different. However, the defensive compounds synthesized by their soldiers are different. A strong chemical polymorphism between sympatric colonies of *R. flavipes* in the SW United States was detected in terms of both the hydrocarbons of the workers and soldiers and in the defensive secretions of the soldiers. The six defensive secretion phenotypes are based on the presence or absence of terpenes whereas the cuticular hydrocarbon phenotypes are based on significant differences in the proportions of the various components. A multivariate analysis (analysis of principal components)

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clearly permitted discrimination of four phenotypes (three in *R. flavipes* and one in *R. santonensis*) without intermediates. The hydrocarbons responsible for these variations were identified, and it was shown that the variations are neither seasonal nor geographic. The phenotypes of the cuticular hydrocarbons (workers and soldiers) and defensive compounds are linked in each colony, forming in three groups in *R. flavipes* Georgia, one subdivided into four subgroups according to the defensive secretion phenotypes. The role of these polymorphisms is discussed and ethological tests indicate that the chemical polymorphism do not determine aggressive behavior. The taxonomic significance of these results is considered and two hypothesis are formulated: (1) We only detected a strong genetic polymorphism in one unique species, and we believe that *R. santonensis* was introduced into Europe in the last century from one *R. flavipes* colony. (2) Chemical variability characterizes the sibling species that can be grouped into the same subspecies *R. flavipes*. Unknown mechanisms of reproductive isolation separate them.

Key Words—Polymorphism, chemotaxonomy, *Reticulitermes flavipes*, *Reticulitermes santonensis*, Isoptera, termites, cuticular hydrocarbons, defensive compounds, terpenes.

INTRODUCTION

In 1792 Rossi described *Reticulitermes lucifugus*. Thereafter, other populations of *Reticulitermes* were identified in southwestern France by different authors such as Latreille (1802) both of whom concluded that they were *Reticulitermes lucifugus*. Later, Boffinet (1842), Bobe-Moreau (1843), De Quatrefages, (1853), and Lespès (1856) indicated that they were, in fact, two distinct species, one of which was close to *lucifugus*. In 1924, Feytaud noted strong morphological differences between termites of southwestern France (Landes, Périgord, and Pyrénées-atlantique), and those from west-central France, near La Rochelle. He described *Reticulitermes santonensis* from the Charente-maritime. In 1958, Buchli described other differences between populations from La Rochelle (*R. santonensis*) and those of southwestern France (*R. (lucifugus) grassei*). Clément (1978) separated *R. santonensis* from *grassei* by demonstrating that these two sympatric taxa do not interbreed and are therefore reproductively isolated. These two species differ in a number of characters, including morphology (Clément, 1978), chemistry (Parton et al., 1981; Baker et al., 1982), and biochemistry (Clément et al., 1981).

Reticulitermes flavipes was described in Austria in 1837 by Kollar, from individuals in a shipment of flowers arriving from the United States. Feytaud (1924) considered *R. flavipes* and *R. santonensis* to be the same species, but other authors, such as Snyder (1926), Lash (1952), Grassé (1954), and Buchli (1958), separated them, although Snyder (1926) had already noted considerable polymorphism of *R. flavipes* in the United States. Becker in 1970 hypothesized

that *R. santonensis* was the result of hybridization between *R. lucifugus* and *R. flavipes*.

Until recently, the different species of the genus *Reticulitermes* have been defined using only morphologic and biometric criteria (size, color, distance between ocelli, and compound eyes of sexuals). In the past few years, biochemical (enzymatic) and chemical (defensive secretions of soldiers and cuticular hydrocarbons) criteria have permitted the separation of the European and American populations. For example, the epicuticular hydrocarbon compositions of the different castes of *R. flavipes* have been described by Howard et al. (1978), and Zalkow et al. (1981) have described the composition of the defensive secretions of the soldiers of *R. flavipes*.

The defensive compounds of European *Reticulitermes* have been described by Parton et al. (1981) and by Lemaire (1982). The epicuticular hydrocarbons of *Reticulitermes*, as well as the chemical signature, were described preliminarily by Clément et al. (1985) and then by Bagnères et al. (1988) and Lange et al. (1989).

Clément distinguished two distinct subspecies of *R. flavipes* in the southeastern United States, using differences in the composition of the defensive compounds of the soldiers (Clément et al., 1985; Clément, 1986). One of these subspecies (*R.f.* 1), also described by Zalkow et al. (1981), possesses two sesquiterpenes, the γ-cadinene and its aldehyde, while *R.f.* 2 lacks the sesquiterpene aldehyde and produces geranyllinalool. This last compound was described for the first time from the defensive secretion of European *Reticulitermes* by Parton et al. (1981) and by Lemaire et al. (1986).

The goal of this work is to compare the chemistry and morphology of the American and French species of *Reticulitermes*, especially *R. flavipes* and *R. santonensis*. This was accomplished by analyzing the cuticular hydrocarbons of the workers and soldiers as well as the defensive compounds secreted by the frontal gland of the soldiers. Hypotheses are presented to explain the polymorphisms and taxonomic values of the two chemical signatures (hydrocarbons and defensive compounds).

METHODS AND MATERIALS

Insects. Seventeen colonies of *Reticulitermes santonensis* were collected in 1986, 1987, and 1988, in southwestern France (Charente maritime, Oléron Island, Coubre forest). Twelve colonies of *flavipes* were collected in the fall of 1987, and 13 in the summer 1988 near Athens, Georgia. The termites were collected from logs housing the colonies, and individuals of each caste were separated and weighed.

Chemistry. Cuticular compounds were extracted for 5 min in 2 ml of pentane (Pestipur). The extracts of soldiers were first chromatographed using Silica Cartridge Sep-Pak (Waters) with an apolar solvent.

Total extracts were analyzed by gas chromatography (GC). The European species (*R.s.*) was analyzed on a Delsi 300 GC with a flame ionization detector (FID) equipped with a fused silica capillary column CP Sil 5 (WCOT) Chrompack (ID 0.25 mm × 25 m). Injection mode was splitless (15 sec). Data were collected on a Enica 10 integrator. Temperature programming was from 30°C to 320°C at 5°C/min for soldiers and 150°C (isothermal 5 min) to 320°C at 5°C/min for workers. Helium was the carrier gas.

The American species (*R.f.*) was analyzed on a Hewlett Packard gas chromatograph (HP 5890A, 5880A, 5790A) connected to HP 3393A, 5880A, 3390A integrators. Two types of SE 54 capillary columns were used: an SE 54 (ID 0.32 mm × 30 m; 0.1 μm of SE 54) for workers, and an SE 54 (idem and 0.3 μm of SE 54) for soldiers. Injection mode was splitless (15 sec). The extracts of this species also were analyzed again in France with the same extracts using the same analytical conditions as employed for *R. santonensis* in order to compare them closely.

Methoxy-mercuration–demercuration followed by GC-MS was carried out to determine the position of double bonds in the alkenes (Plattner et al., 1976; Blomquist et al., 1980; Lange et al. 1989).

The quantification of products was performed using an internal standard (nC21: heneicosane) added before the Sep-Pak separation for soldier analysis. Coefficients (GC response factors) were calculated for each peak, using a mixture of pure compounds (nC16–nC40), depending on the sensitivity of the detector (FID) for each kind of hydrocarbon at various concentrations.

To separate alkanes, monoenes, and dienes, thin-layer chromatography (TLC) was carried out on silica gel plates treated with 10% silver nitrate ($AgNO_3$) and developed in 8% benzene–92% hexane.

Each fraction from the two species was analyzed by GC and GC-MS on a Nermag R 1010C connected with a Sidar 8 computer, using electron impact (70 eV) and chemical ionization with CH_4 and NH_3 (100 eV, 0.1 torr).

Quantities and percentages of components were calculated with a Lotus Software program (Lotus 123, v.2.01) for each peak adjusted with the GC response factor. Results were analyzed by computer IBM-PC AT3 using Statgraphics Software for the Summary Statistics and for multivariate methods (principal component analysis).

Multivariate Method. Inter- and intraspecific variations were studied by principal component analysis. The variables were comprised of the 21 (22 for the soldiers) epicuticular hydrocarbons. The analysis was performed on 42 extracts of *R. santonensis* of which 17 were of 20 workers, 7 were of about 10 soldiers, and 11 of different castes found in the sampled colonies (male and female sexuals, neotenics of both species, nymphs, and eggs).

Fifty-three extracts of *R. flavipes*, of which 25 were of workers, 24 of soldiers, and one of each of the castes were analyzed. These included neotenics,

nymphs, sexuals, and white soldiers (or presoldiers). In all, components from 95 samples were compared individually in different matrices (entire species, or workers and soldiers separately, one species or the two simultaneously).

The variables were normalized automatically during the principal component analysis for a better comparison. Correlation analysis was performed to identify highly correlated compounds. So that some of these could be eliminated, principal component analysis was then carried out first on all the compounds and then on the compounds belonging to certain chemical families (linear alkanes, branched alkanes, etc.), and then on selected compounds based on these analyses. In this way, we were able to eliminate differences in sample sizes.

Multidimensional analysis permitted us to project vector matrices on first, second, or third axes, and to visualize the inertia of each variable on these axes. Only the first five axes were studied since they account for 80% of the variation. Different approaches were applied to determine which character (i.e., hydrocarbons) contributes the most to the separation of the different groups (castes, seasons, phenotypes).

Behavioral Tests. Behavioral tests were performed with the same nests used in the chemical analysis (*R. santonensis* and *R. flavipes*): 20 termites from one colony of *R.s.* or *R.f.* were mixed with 20 of another colony of others *Reticulitermes* species in each Petri dish containing humid filter papers. There were five replicates for each experiment. Twenty-four hours later, an aggression rating was calculated using the formula of Clément (1985): $Ag = (M + m/2) * 2.5$ (M = mean number of dead termites, m = mean number of injured termites). If the index reached 25, the two nests were considered to be aggressive.

Biometric Study. Three measurements were taken on scanning electron microscope photographs (Figure 1). These photographs show the head of the workers of the two species and the different phenotypes. About a dozen individuals were examined for each phenotype (except D). The head was photographed in a position that gave a clear view of the postclypeal region. The measurements L, AB, and BC, were taken after verification that the tangent T appeared horizontal on the screen. The photograph included here shows our three measurements (L, AB, BC) and the tangent T. The research of Clément (Clément, 1978, Clément et al., 1982) served as a reference enabling us to obtain clear results. The measurements were taken in millimeters, and the mean $\pm$SD was calculated for N samples. Tables 5 and 6 below present the results.

We used the following formula to calculate our index:

$$(\text{distance AB} + \text{distance BC}) \times \text{length L}$$

Tangent T served as a point of departure for the vertical measurements. Tangent T′ served for measurement L, and T″ was made using as a reference

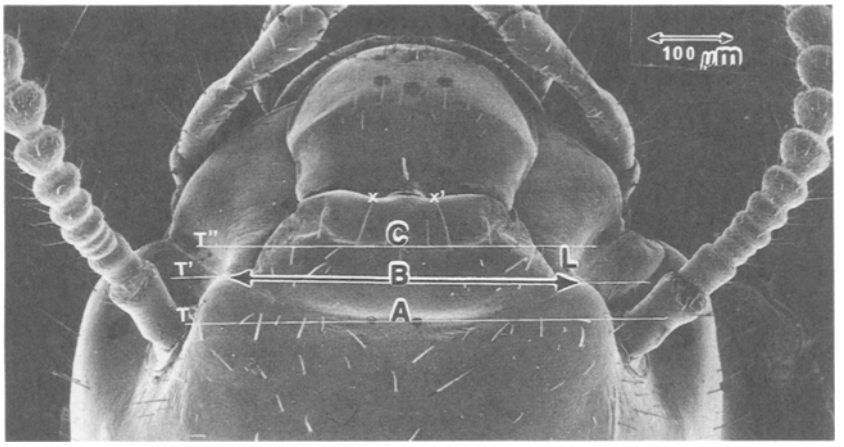


FIG. 1. Scanning electron microscope photography of a worker head with the three measurements used in biometric study.

the bristles x and x', which were always present. The measurements AB and BC were done separately, because the sum AB + BC, although very interesting, was not sufficient since each measurement was significant. Thus there are three measurements for each individual.

RESULTS

Defensive Compounds of Soldiers

R. santonensis. The constituents of the frontal gland of soldiers of *R. santonensis* were initially described by Parton et al. (1981) and subsequently analyzed in detail by Lemaire et al. (1986). The main components are terpenes of three different types: monoterpenes (α-pinene, β-pinene, and limonene), unidentified sesquiterpenes (small quantities), and a diterpene alcohol, geranyllinalool, in moderate but variable quantities (30–500 ng/individual).

R. flavipes. Based on the composition of the defensive compounds, this species exhibits six phenotypes (A–F). The different variations were observed in November and in June, in a small area near the campus of the University of Georgia (around 20 km away).

Group A possesses the same three monoterpenes as *R. santonensis* (α- and β-pinene, and limonene) and two sesquiterpenes (γ-cadinene and its aldehyde) (Zalkow et al., 1981). This group corresponds to *R. flavipes* (*R.f.* 1) described by Clément (1986). Two colonies collected in November 1987 and one in June 1988 exhibited this phenotype.

Group B is similar to group A but lacks the monoterpenes and chemically resembles *flavipes* described by Zalkow et al. (1981). It was found twice in November and once in June.

Group C possesses the monoterpenes, the two sesquiterpenes in equivalent amounts (about 800 ng/insect), and the same diterpene alcohol (geranyllinalool) as *R. santonensis* in large quantities (500–3000 ng/insect). This group is the most often encountered among our collections (three of 12 colonies in November and six of 13 in June).

Group D is identical to group C, but lacks the monoterpenes (found twice in November, and three times in June).

Groups E and F possess geranyllinalool in quantities equivalent to groups C and D, but γ-cadinene is completely absent and the aldehyde seems chemically unstable, possibly due to the lack of the other sesquiterpene, γ-cadinene. E does not possess monoterpenes (found three times in November, and once in June). F possesses the three monoterpenes and was collected only once in June 1988 (probably *R.f.* 2 in Clément et al., 1985).

Conclusions. R. santonensis is homogeneous, and *R. flavipes* possesses many polymorphisms in its defensive chemistry. Thus, between these two species, we can characterize six sympatric phenotypes and one allopatric phenotype (*R.s.*). The seven chromatograms of the different phenotypes are presented in Figure 2 and are summarized in Table 1. We have also observed several additional terpenoid compounds in very small quantities, in particular sesquiterpenes, but their occurrence was highly inconsistent and these results are not analyzed.

Cuticular Hydrocarbons

Workers of R. santonensis. Only peaks with quantities greater than 0.5 ng/insect were quantified and numbered (Figure 3A). We found 21 peaks situated between nC22 (only in trace amounts) and nC26 (a21). TLC–$AgNO_3$ separated the saturated alkane fraction from the monoenes. In this second fraction, three monoenes (forming 20% of the determined cuticular signature) were isolated, and GC-MS analysis (after methoxy derivatization) determined that they were 9-tricosene (e1) (Figure 4: mass spectrum of 9-C23 : 1), 9-tetracosene (e6) (the two were always present but in small quantities), and 9-pentacosene (e12) present in large quantities. Coinjection with standard monoenes established e1, e6, and e12 as *cis* isomers. The quantities, expressed in nanograms per milligram of insect and the percentages of all the peaks are given in Table 2.

Thin-layer chromatography separated out a last fraction containing dienes. One diene comprised about 7% of the cuticular hydrocarbons and was quantified and identified as 7,9-pentacosadiene (n17) after methoxy-mercuration–demercuration.

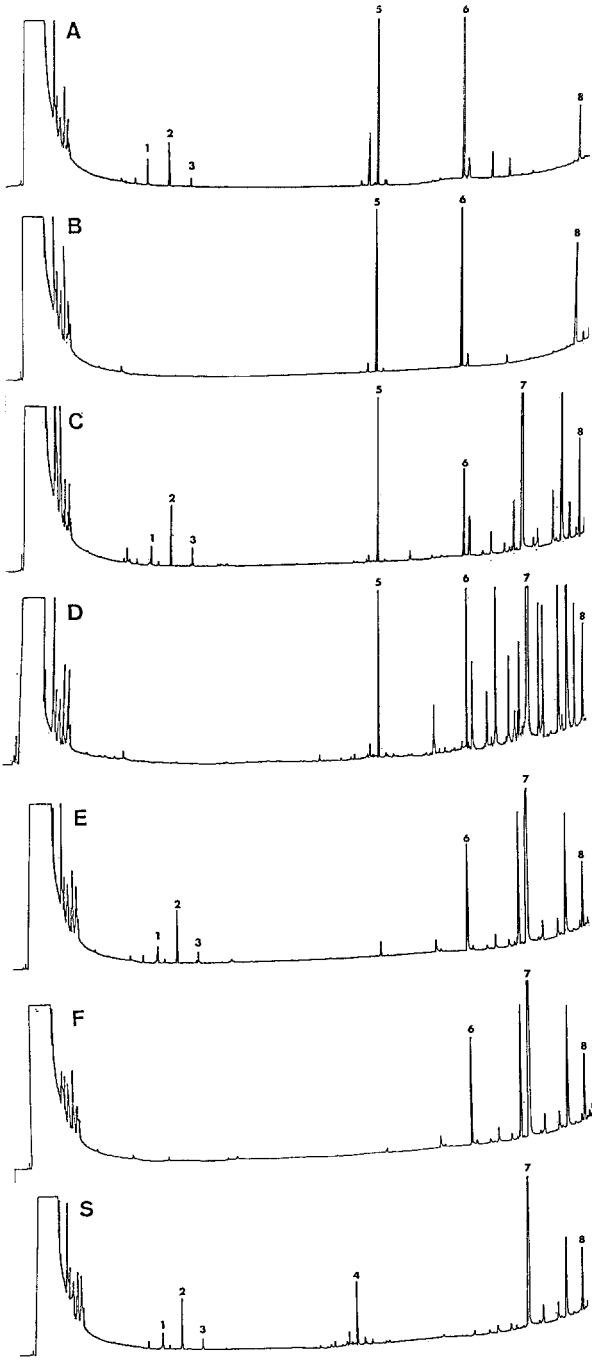


FIG. 2. Gas chromatograms of the six phenotypes of soldiers in *Reticulitermes flavipes* (A–F) and in *R. santonensis* (S) listed in Table 1. 1: α-pinene, 2: β-pinene, 3: limonene, 4: unknown sesquiterpene 5: γ-cadinene, 6: aldehyde cadinene, 7: geranyllinalool, 8: n-C23.

TABLE 1. DEFENSIVE COMPOUNDS PHENOTYPES OF *R. flavipes* AND *R. santonensis*

	Phenotypes						
	R. flavipes						*R.s.*
Terpenes	A	B	C	D	E	F	S
α-Pinene	yes		yes			yes	yes
β-Pinene	yes		yes			yes	yes
Limonene	yes		yes			yes	yes
γ-Cadinene	yes	yes	yes	yes			
Aldehyde cadinene	yes	yes	yes	yes	yes	yes	
Geranyl linalool			yes	yes	yes	yes	yes
Sesquiterpene							yes

Three linear alkanes were quantified, in addition to hexacosane (nC 26): nC23 (a3), nC24 (a3), and nC25 (a14). Approximately 30% of the cuticular hydrocarbons were in this fraction.

Monomethylalkanes were the most abundant compounds found (about 50% of the total). These consisted of 11-methyl (m4) and 4/2-methyl (m5) tricosane (4- or 2-Me-branched), 12-methyl (m9) and 4/2-methyl (m11) tetracosane, a blend of 11-Me and 13-methylpentacosane (m16), and, finally, 4/2 Me(m18) and 3 Me(m 19) pentacosane. The mass spectra of 11-MeC23 (Figure 5) and 11-Me + 13-MeC25 (Figure 6) as well as the equivalent chain length (ECL) of each peak with diagnostic ions (EI and CI) (Table 3) are included here.

Several small peaks forming 25% of all the peaks were identified as 9-C23 : 1 (e2), almost certainly the isomer of peak e1 (Z,9-C23 : 1), 3-MeC23 (m7), x-C25 : 1 (e13), probably the *trans* isomer of peak e12 (Z,9-C25 : 1) and 5,17-diMeC25 (d20). Peak ×15 was not identified and comprised about 0.45% of the total of the cuticular hydrocarbons of the workers.

Workers of R. flavipes. Coinjections were made with extracts of *R. santonensis* workers (and with a series of standards). Twenty-one peaks were redetermined by GC-MS and then quantified, and their retention times were calculated and compared to those found for *R. santonensis* (chromat. *R. flavipes* Figure 3B) (Howard et al., 1978). The mass spectra of the hydrocarbons were identical to those found for *R. santonensis*. Mixtures of the hydrocarbons of *flavipes* and *santonensis* were therefore qualitatively identical. The proportions of the two species were compared by means of Student's *t* test (α: 0.5%) (Table 2).

The quantitative differences were significant, particularly the blend of 11- and 13-methylpentacosane and 7,9- pentacosadiene. We found 54% monomethyl-branched hydrocarbons and only 2% dienes in *R. flavipes*, compared to 42% and 7%, respectively, in *R. santonensis*. The largest differences were in

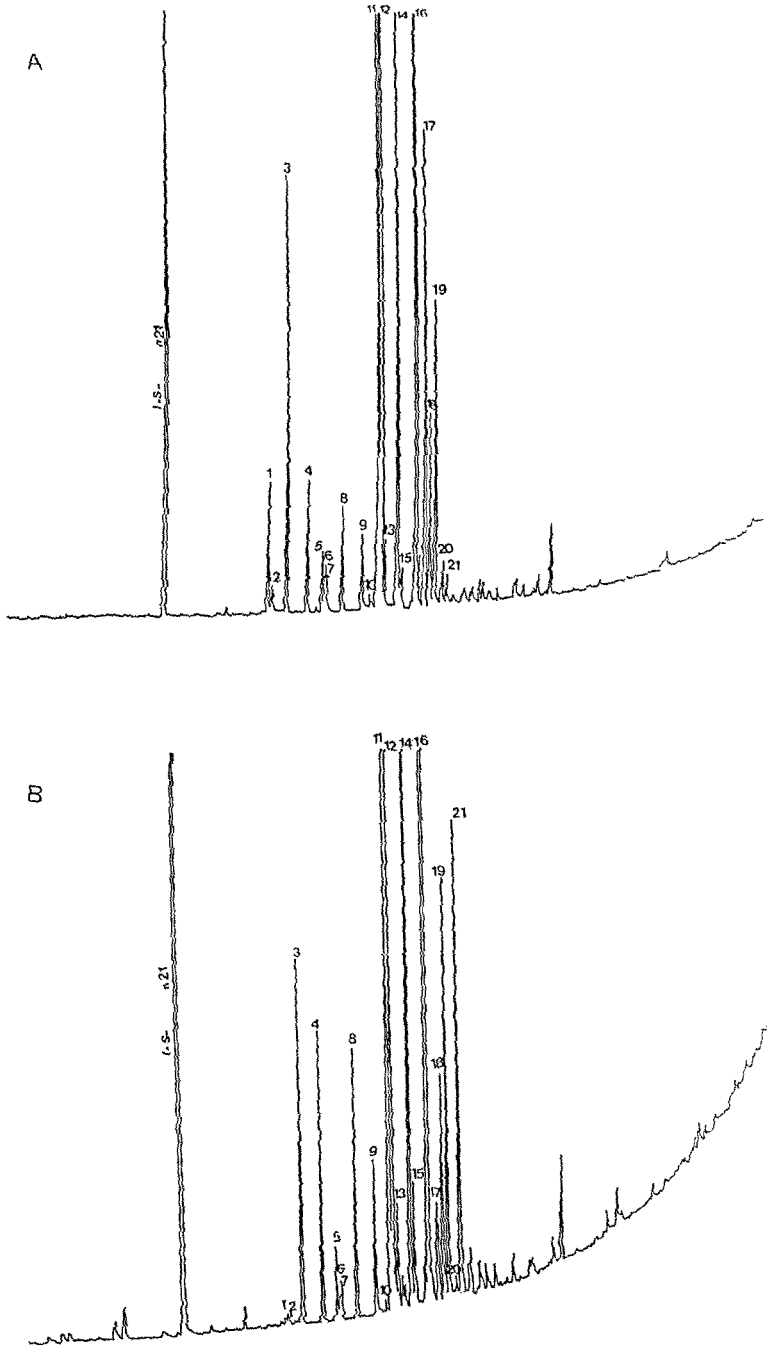


FIG. 3. Gas chromatograms of cuticular hydrocarbons of *Reticulitermes santonensis* (A) and *R. flavipes* (B) workers.

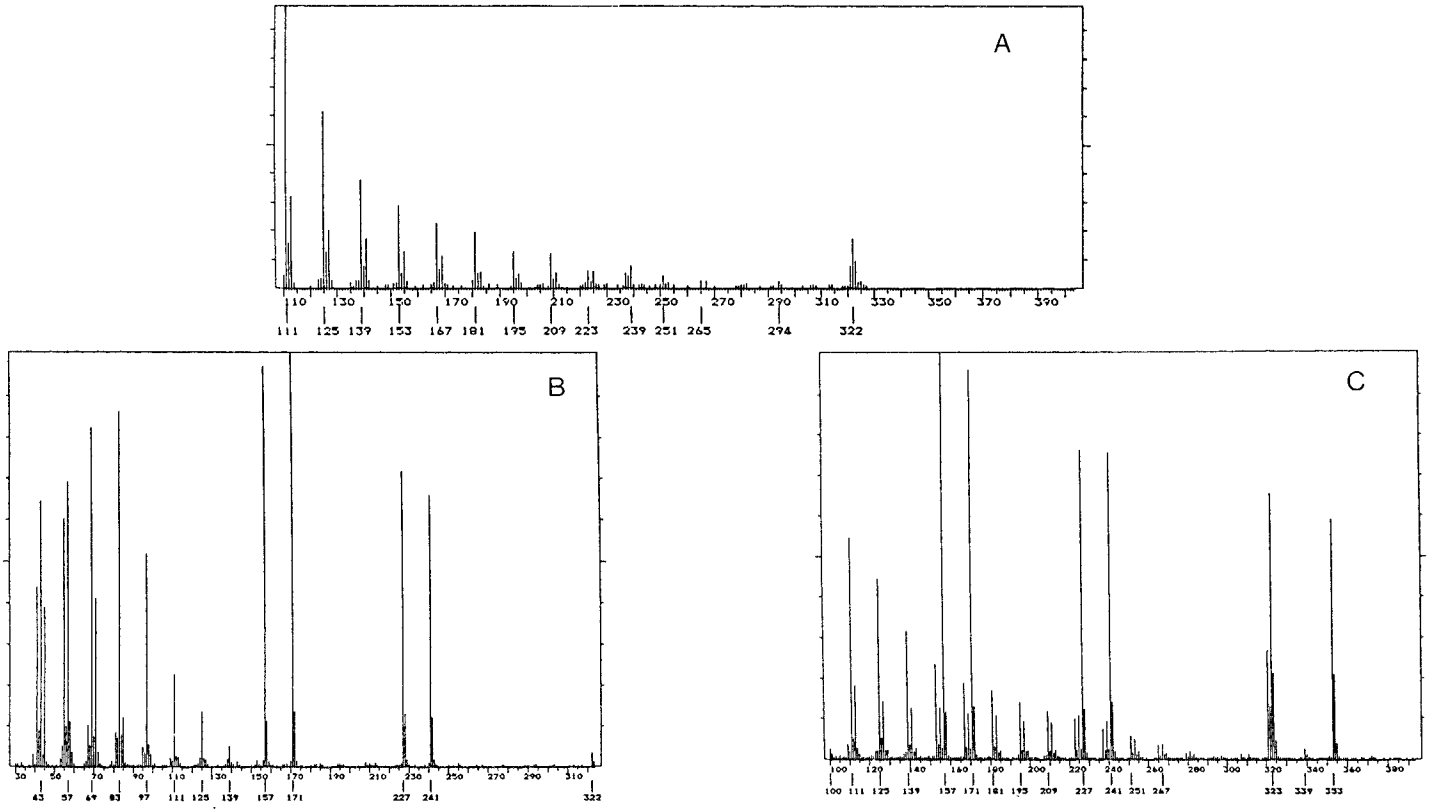


FIG. 4. Mass spectrum of peak 1 (el): 9-tricosene, $ClCH_4$ (A), 9-methoxy and 10-methoxy tricosene: El (B), and $ClCH_4$ (C). The figure shows the $ClCH_4$ mass spectrum 9-tricosene (el), which gives a molecular ion and a typical alkene fragmentation pattern that does not permit the assignment of the position of the double bond. Applications of methoxymercuration–demercuration to el produced a mixture of two monomethoxy derivatives, 9- and 10-methoxytricosane. The EI mass spectrum (B) of the methoxylated derivatives yielded major fragment ions at *m/z* 241 and 137 for the 9-methoxy compound and *m/z* 227 and 171 for the 10-methoxy derivative. The $ClCH_4$ mass spectrum yielded a MH^+ ion at *m/z* 333, a strong M − MeOH ion and major fragment ions discussed about. Using the above methodology other alkenes were identified to be 9-tetracosene (e6) and 9-pentacosene (e12).

TABLE 2. PERCENTAGES AND QUANTITES OF HYDROCARBONS FROM *Reticulitermes santonensis* AND *R. flavipes*, AND STATISTICAL DIFFERENCES[a]

		Percentages						Quantities			
		Workers			Soldiers			Workers		Soldiers	
Peak[b]	Identification	*R. sant.*	*R. flav.*	*Stat.*	*R. sant.*	*R. flav.*	*Stat.*	*R. sant.*	*R. flav.*	*R. sant.*	*R. flav.*
el	Z,9-tricosene	2.25	0.57	*	2.15	0.82	*	2.29	0.31	11.49	2.45
		(0.38)	(0.63)		(0.67)	(0.96)		(1.63)	(0.45)	(5.03)	(5.78)
e2	*x*-tricosene	0.35	0.20	*	0.51	1.22	NS	0.36	0.11	2.74	3.64
		(0.23)	(0.18)		(0.10)	(1.36)		(0.22)	(0.18)	(1.55)	(3.94)
a3	*n*-tricosane	7.98	5.45	*	7.18	6.03	NS	8.11	2.97	38.31	18.07
		(1.80)	(1.96)		(1.47)	(3.25)		(5.19)	(1.77)	(19.39)	(9.66)
m4	11Me-tricosane	1.59	2.31	*	2.55	3.16	NS	1.62	1.26	13.63	9.45
		(0.36)	(1.05)		(0.81)	(1.69)		(0.86)	(0.84)	(8.43)	(7.77)
e4/5	insat. unknown	0.00	0.00		0.29	0.04	*	0.00	0.00	1.56	0.12
					(0.15)	(0.09)				(1.21)	(0.39)
m5	4/2Me-tricosane	1.17	1.05	NS	0.76	1.34	*	1.19	0.57	4.05	4.01
		(0.43)	(0.43)		(0.10)	(0.36)		(0.76)	(0.40)	(1.95)	(3.40)
e6	Z,9-tetracosene	0.47	0.33	NS	1.30	0.36	*	0.48	0.18	6.94	1.09
		(0.35)	(0.26)		(0.23)	(0.54)		(0.39)	(0.14)	(4.10)	(2.83)
m7	3Me-Tricosane	0.46	0.66	NS	0.55	0.74	NS	0.47	0.36	2.94	2.22
		(0.35)	(0.43)		(0.10)	(0.35)		(0.41)	(0.31)	(1.78)	(1.46)
a8	*n*-tetracosane	2.30	3.03	*	1.22	1.59	NS	2.34	1.65	6.52	4.76
		(0.63)	(0.58)		(0.56)	(0.46)		(1.42)	(0.92)	(3.62)	(2.10)
m9	11Me-tetracosane	1.19	1.83	*	1.48	2.50	*	1.21	1.00	7.89	7.48
		(0.25)	(0.56)		(0.17)	(0.93)		(0.68)	(0.74)	(4.21)	(5.76)
m10	5Me-tetracosane	0.30	0.04	*	0.60	0.22	*	0.31	0.02	3.20	0.66
		(0.05)	(0.12)		(0.23)	(0.20)		(0.25)	(0.09)	(1.01)	(0.88)

m11	4/2Me-tetracosane	10.42	9.72	NS	15.55	11.40	NS	10.59	5.30	82.96	34.14
		(1.83)	(1.75)		(6.15)	(3.97)		(6.57)	(3.11)	(54.15)	(19.84)
e12	Z-9-pentacosene	18.44	12.75	*	25.16	13.03	*	18.75	6.95	134.27	39.02
		(4.19)	(8.58)		(5.71)	(11.9)		(9.51)	(4.79)	(79.55)	(66.64)
e13	*x*-pentacosene	0.59	1.45	*	0.29	0.29	*	0.60	0.79	1.55	0.87
		(0.32)	(0.72)		(0.26)	(0.32)		(0.36)	(0.52)	(1.50)	(1.06)
a14	*n*-pentacosane	16.78	13.88	NS	7.10	8.69	NS	17.06	7.57	37.90	26.03
		(3.80)	(3.05)		(3.35)	(4.03)		(11.78)	(4.67)	(18.34)	(12.14)
x15	unknown	0.38	1.10	*	0.58	1.34	*	0.39	0.60	3.12	4.00
		(0.35)	(0.55)		(0.40)	(0.98)		(0.45)	(0.43)	(2.25)	(2.23)
m16	11+13Me-pentacos.	19.94	29.71	*	19.43	33.47	*	20.27	16.20	103.70	100.25
		(2.79)	(8.45)		(3.51)	(13.8)		(12.36)	(10.78)	(59.18)	(83.87)
n17	7,9-pentacosadiene	6.98	1.76	*	8.52	2.22	*	7.10	0.96	45.49	6.66
		(1.99)	(2.02)		(2.45)	(3.10)		(5.28)	(0.93)	(27.37)	(6.49)
m18	4/2Me-pentacosane	2.64	3.28	NS	1.50	2.57	*	2.68	1.79	8.00	7.69
		(0.32)	(0.86)		(0.20)	(1.07)		(1.48)	(1.34)	(3.61)	(5.48)
m19	3Me-pentacosane	4.55	5.56	*	1.88	4.11	*	4.63	3.03	10.04	12.32
		(0.77)	(1.06)		(0.30)	(1.24)		(2.72)	(2.13)	(4.52)	(7.46)
d20	5,17diMe-pentacosane	0.74	0.35	*	0.66	0.72	NS	0.75	0.19	3.53	2.15
		(0.42)	(0.58)		(0.33)	(0.90)		(0.69)	(0.37)	(2.19)	(2.58)
a21	*n*-hexacosane	0.45	4.99	*	0.71	4.14	*	0.46	2.72	3.78	12.41
		(0.43)	(3.00)		(0.67)	(3.07)		(0.28)	(3.49)	(1.99)	(19.78)
								101.66	54.53	533.61	299.49
	Qt total							(53.45)	(32.61)	(260.21)	(180.27)

[a] Student's *t* and Fisher tests. Asterisk indicates significant difference (α = 0,5) between *R.s.* and *R.f.* for workers and soldiers. N.S. indicates no significant difference. Standard deviation is in small letters in parenthesis and quantites are in ng/mg insect.

[b] a = n. alkane, e = monosene, n = diene, m = monomethyl branched, d = dimethyl branched, and x = unknown.

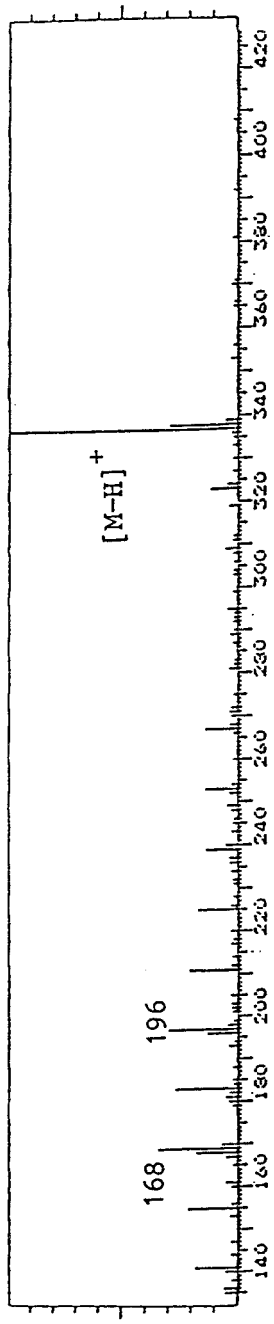


FIG. 5. $ClCH_4$ mass spectrum of peak m4 identified as 11-MeC23.

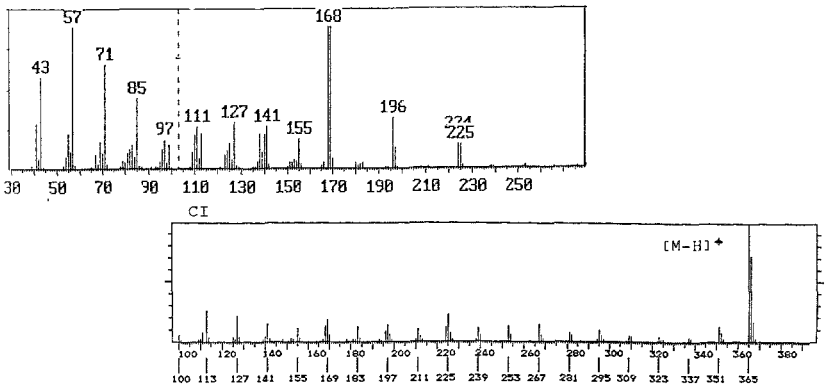


FIG. 6. EI and $ClCH_4$ mass spectrum of m16 identified as 11-methyl and 13-methyl pentacosane.

TABLE 3. HYDROCARBONS IDENTIFIED BY GC-MS EI AND CI FOR *R. santonensis* AND RE-IDENTIFIED FOR *R. flavipes*.

Code peak	Compound	CN[a]	ECL[b]	Diagnostic	EI-MS	CI-MS
e1	*z*,9-C23 : 1	23	22.66		322	323
e2	*x*-c23 : 1	23	22.74		322	323
a3	*n*-C23	23	23.00		324	323
m4	11Me-C23	24	23.37	168/169,196/197	338	337
m5	4/2Me-C23	24	23.64	294/295	338	337
e6	*Z*,9-C24 : 1	24	23.69		336	337
m7	3Me-C23	24	23.72	308/309	338	337
a8	n-C24	24	24.00		338	337
m9	11Me-C24	25	24.35	168/169,210/211	352	351
	(+12Me-C24)			182/183,196/197	352	351
m10	5Me-C24	25	24.53	294/295	352	351
m11	4/2MeC24	25	24.65	308/309	352	351
e12	*Z*,9-C25 : 1	25	24.71		350	351
	(+*x*,y-C25 : 2)	25			348	349
e13	*x*-C25 : 1	25	24.79		350	351
a14	*n*-C25	25	25.00		352	351
x15	x		25.06			
m16	13+11Me-C25	26	25.36	196/197	366	365
				168/169,224/225	366	365
n17	7,9-C25 : 2	25	25.55		348	349
	(+5Me-C25)	26		308/309	366	365
m18	4/2Me-C25	26	25.64	322/323	366	365
m19	3Me-C25	26	25.75	336/337	366	365
d20	5,17Dime-C25	27	25.91	85,140,267,323	394	393
a21	*n*-C26	26	26.00		380	379

[a] CN = carbon number.
[b] ECL = equivalent chain length.

the quantities that were almost twice as much in *R. santonensis* (102 ng/mg of insect) as in *R. flavipes* (55 ng/mg of insect) (Table 2).

Soldiers of Both Species. After chromatography on the Sep-Pak microcolumn, 22 peaks were identified in the nonpolar fraction, of which 21 were identical to those of the workers. The remaining peak fell between 11Me-C23 (m4) and 4Me-C23 (m5) and came off on the thin-layer plate (TLC–$AgNO_3$: 10%) with the monoenes. It is designated e4/5 (ECL = 23.5) and could not be determined by GC-MS; it is not very abundant in the two species (see Figure 7).

For both species the quantities of the cuticular hydrocarbons in the soldiers are twice as great as those of the workers, or 534 ng/mg insect for *R. santonensis* and 299.5 ng/mg for *R. flavipes* (see Table 2).

Conclusions. The epicuticular hydrocarbons compounds are chemically identical in the two species. However, their proportions differ and they are present in different quantities. The substances are the same for all castes of *R. santonensis* and *R. flavipes*, where only the relative proportions of their mixtures vary (Table 4). Some minor peaks appear after peak nC26 (a21), and their identities (hydrocarbons or terpenes) were not determined.

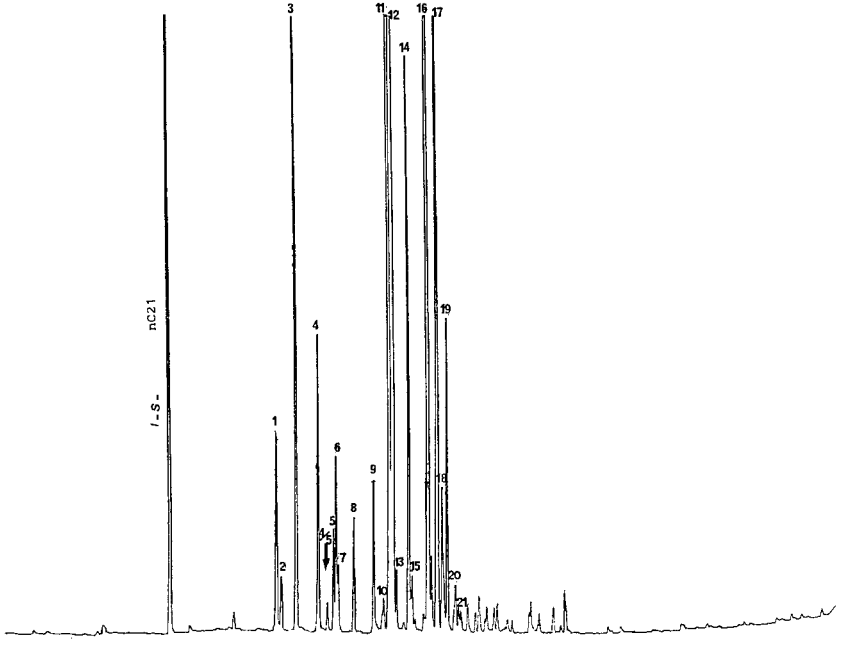


FIG. 7. Gas chromatogram of cuticular hydrocarbons of soldiers of both species. Peaks are listed and described in Tables 2 and 3.

TABLE 4. RELATIVE PROPORTIONS OF HYDROCARBON FAMILIES OF *R. santonensis* AND *R. flavipes*

	Workers		Soldiers	
	R. santonensis	*R. flavipes*	*R. santonensis*	*R. flavipes*
n-Alkanes	27.51%	27.35%	16.21%	20.45%
Mono-branched	42.26%	54.16%	44.30%	59.51%
Di-branched	0.74%	0.35%	0.66%	0.72%
Monoenes	22.10%	15.30%	29.70%	15.76%
Dienes	6.98%	1.76%	8.52%	2.22%

Multivariate Analysis of Cuticular Compounds

Variation in Soldiers of Two Species (Figure 8). After principal component analysis of the different cuticular compounds expressed as relative proportions, four groups of soldiers can be distinguished when component canonical axes 1 and 2, which account for most of the variability (>50%), are plotted.

Each extract (phenotype) is represented as a point in the three-dimensional principal component space (C) (Figure 8A). On the second plotting, the cuticular variables (hydrocarbons) are represented as a point (here at the top of a vertical line) in a three-dimensional component weight space (*W*) (Figure 8B).

The first axis accounts for 35% of the variability and is determined by the following compounds: In positive values, all alkenes 9-C23:1 (e1), 9-C24:1 (e6), 9-C25:1 (e12), e4/5, the diene 7,9-C25:2 (n17), 4/2Me-C24 (m11), nC23 (a3), nC25 (a14), 5Me-C24 (m10), x-C25:1 (e13) and 5,17diMe-C25 (d20). Soldiers of groups A and B of *flavipes* and groups (*R.s.*) can be distinguished by these hydrocarbons.

In negative values, most of the methyl compounds, such as 11Me-C23 (m4), 12Me-C24 (m9), 11 + 13Me-C25 (m16), 4/2Me-C23 (m5), 4/2Me-C25 (m18) and 3Me-C25 (m19), a linear alkane nC26 (a21) and peaks m7 (3Me-C23), e2 (×9-C23:1) and ×15, the *R. flavipes* soldiers groups (C, D, E, F) are separated from groups A and B. The major compounds discriminating the group (C, D, E, F) consist of a mixture of 11- and 13-methylpentacosane (m16).

The first axis, then, separates soldiers belonging to groups A + B (*R.f.*) and S (*R.s.*), from those belonging to C, D, E, F (*R.f.*).

The second axis accounts for 15% of the variability and allows the separation of the following: For positive values, group A (*R.f.*), principally by the diene 7,9-C25:2 (n17), the 5,17-dimethyl-C25 (d20), and the *n*-pentacosane (a14). This phenotype is chemically very similar to phenotype S (*R.s.*). For

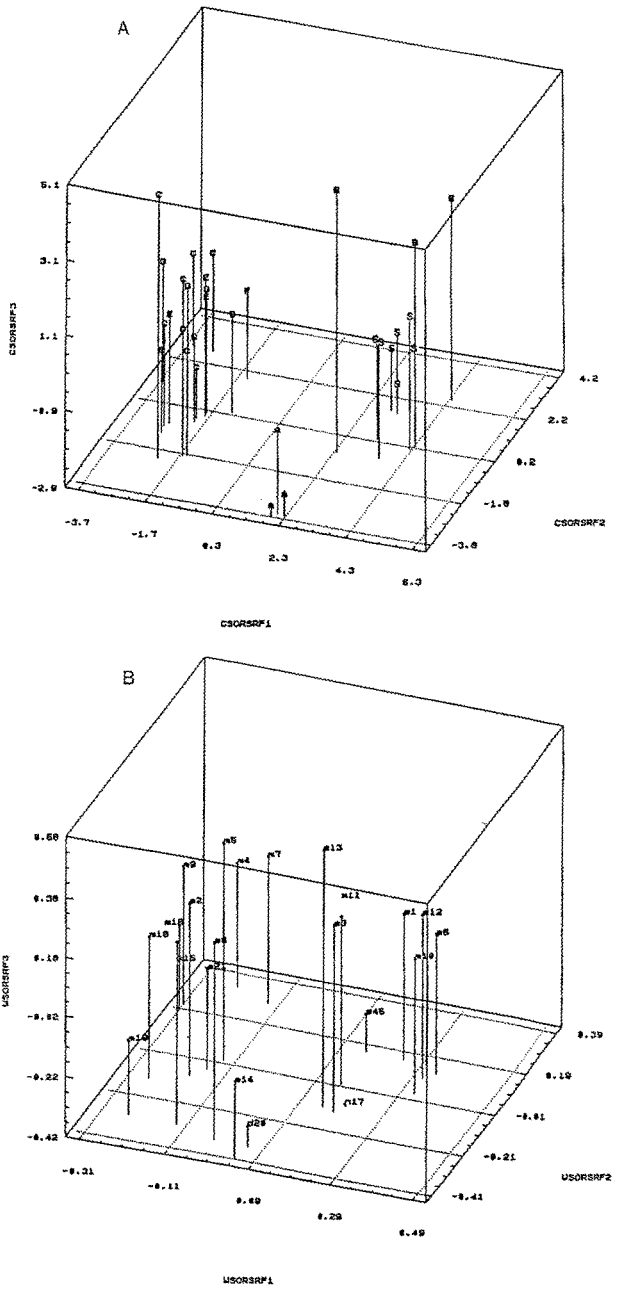


FIG. 8. Plot of the three axis of the principal component analysis of soldiers *Reticulitermes flavipes* and *R. santonensis*. (A) Plot of the seven phenotypes (A–F for *R. flavipes*, S for *R. santonensis*) in the principal component space (CSORSRF). (B) Plot of the 22 codes of the cuticular variables expressed as relative proportions in the component weight space (WSORSRF).

negative values, phenotype B is distinguished principally by *n*-tricosene (a3), but also by 9-tetracosene (e6), 9-pentacosene (e12), 9-tricosene (e1) and 5Me-C25(m10). Group B is mingled with phenotype S, when all of the products in the matrix are used.

Phenotype S, corresponding to *R. santonensis*, is discriminated by compounds often shared in common with groups A and B, such as 9-pentacosene (e12), 7,9-pentacosadiene (n17), e4/5, and 9-tetracosene (e6). The compound d20 (5,17diMe-C25) seems to be the most important in distinguishing *R. santonensis* from phenotypes A and B.

Axis 3 accounts for 11% of the variability and very clearly separates phenotype B from *R. santonensis*, due to the inertia of the following hydrocarbons: 9-C24:1 (e6), 9-C25:1 (e12), and 5Me-C24(m10).

Phenotypic variation in workers of R. santonensis *and* R. flavipes *(Figure 9).* The same groups are found distributed along the different axes as those found in the soldiers (one point for one extract, in the two-dimensional space). Note that the vector plots of the workers (proportions of their cuticular hydrocarbons) of different colonies, especially in *R. flavipes*, correspond to plots of the defensive compounds of nestmate soldiers. The cuticular compounds distinguishing the different phenotypes were slightly different from those of the soldiers.

Axis 1 accounted for 34% of the variability. The dispersion of the phenotypes was, CDEF in negative values and ABS in positive values of axis 1 (principal component space, Figure 9A). A plot of the different vectors on this axis shows the same grouping: methylated compounds and monoenes plus diene on each side of this axis for the component weight space (Figure 9B).

Axis 2 accounted for 14% of the variability and again separates workers of colonies with phenotype A (positively) from those of colonies with phenotype B (negatively).

The following compounds distinguish workers in both axes of W (component weight) space in group A: 7-9:C25 (n17) and 7,17diMe-C25 (d20); in group B: nC23 (a3) and 4/2Me-C23 (m5); workers of group S (*R. santonensis*): 9-C25:1 (e12), 9-C24:1 (e6), 5Me-C24 (m10); lastly, 11Me + 13Me-C25 (m16) and 3Me-C25 (m19) for workers (CDEF) (negative values).

Discrimination of different castes of R. flavipes *(Figure 10).* A plot of the first axis (25% of the variability) and of the second axis (20% of the variability) of the principal components (C) and of the component weight (W), separates the different phenotypes into three groups identical to the preceding analysis: on one side of axis 1 (positive) the group composed of phenotypes CDEF, and on the other side (negative), the phenotypes A and B again separated along axis 2.

The first axis distinguishes equally the castes of soldiers A and B from the other castes of the same phenotype. Soldiers of group B are distinguished in the

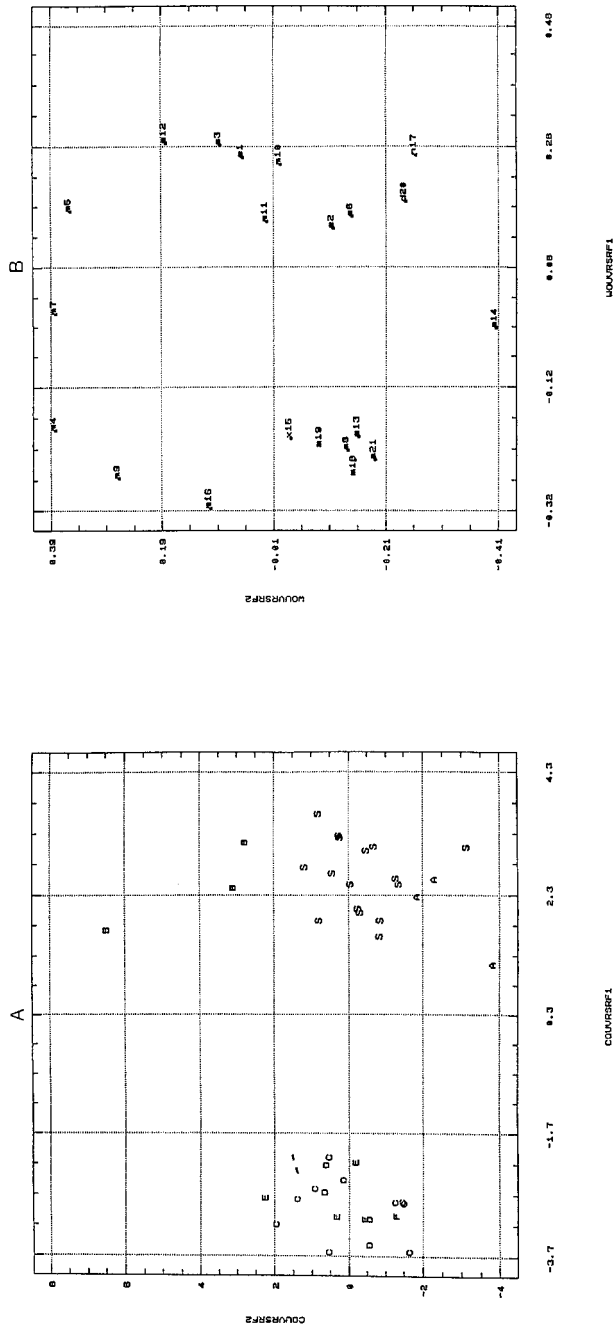


FIG. 9. Plot of the two first axis of the principal components analysis of workers *Reticulitermes flavipes* and *R. santonensis* using proportion of cuticular hydrocarbons. (A) Plot of six phenotypes of *R. flavipes* (A–F) and the phenotype *R. santonensis* (S), in the principal components space (COUVRSRF). (B) Plot of the 21 cuticular variables (codes) in the component weight space (WOUVRSRF).

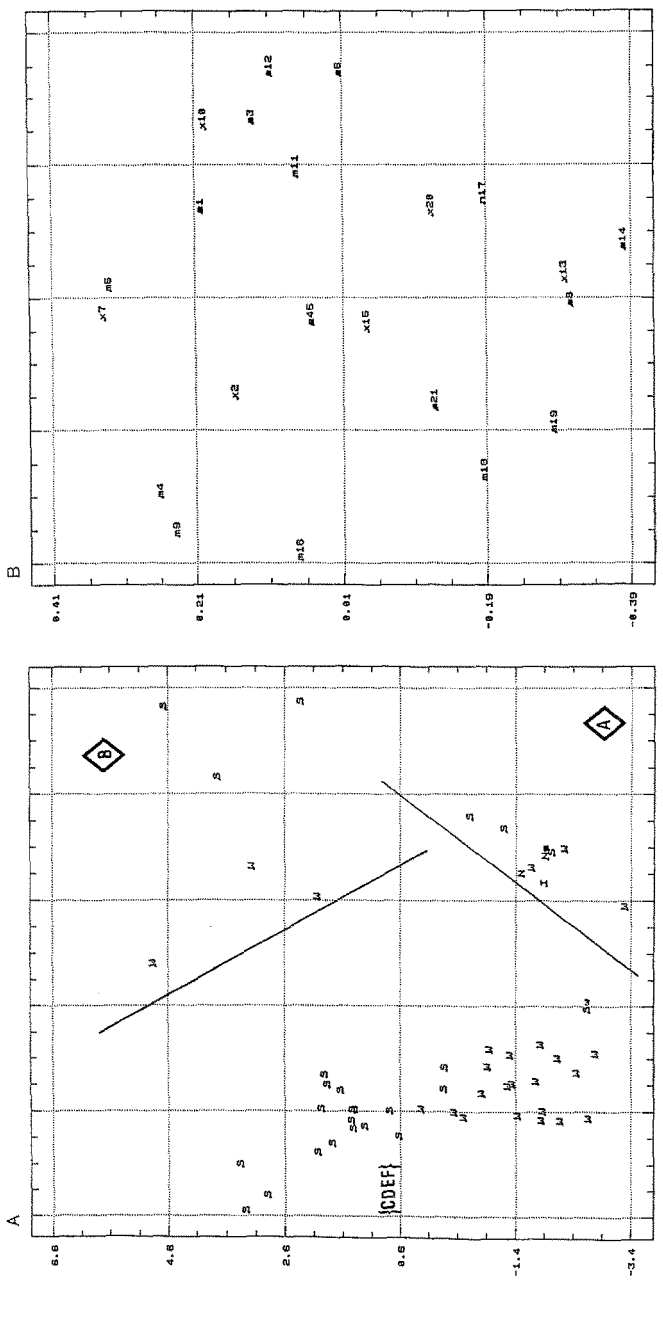


FIG. 10. Plot of the two first axes after principal component analysis of proportion of cuticular compounds of different castes of *Reticulitermes flavipes*. (A) Principal component space (CRF) with different castes of different phenotypes A, B, CDEF. (B) Component weight space (WRF) with the 22 cuticular variables. Castes are S = soldier, W = worker, Ns = nymph small covers, SW = white soldier, N = neotenic, I = imago.

space W (component weight) by the compounds 5Me-C24 (m10), nC23 (a3), 9-C25 : 1 (e12), and 9-C24 : 1 (e6) from B workers in which the following compounds contribute to their grouping: 4/2Me-C24 (m11), 9-C23 : 1 (e1), 4/2Me-C23 (m5), and 3Me-C23 (m7).

Soldiers of group A are distinguished by 7,9-pentacosadiene (n17), 5,17diMe-C25 (d20), and 9-C24 : 1 (e6), which are opposed to the blend m16 (11 + 13Me-C25) along axis 1. Workers of group A are distinguished by the compounds x-C25 : 1 (e13), nC25 (a14), and nC24 (a8).

The second axis also distinguishes soldiers CDEF from the other castes of the same group (CDEF) (one point for one phenotype for the C space, principal component).

The compounds contributing to this discrimination, in the W space (component weight), are of one part, 11Me-C24 (m9), 11Me-C23 (m4) and 11 + 13Me-C25 (m16) for the soldier caste (CDEF); and of an other part, 4/2Me-C25 (m18), 3Me-C25 (m19), and nC26 (a21), for the other castes (primarily those of the workers).

Seasonal variations in R. flavipes *workers (Figure 11).* The colonies of *R. flavipes* were collected at two different times: November 1987 (fall, collection 1) and June 1988 (summer, collection 2). Axis 3 of the principal component analysis of the workers of *R. flavipes* (10% of the variability) distinguishes the extracts (one point for one extract) of the workers from collections 1 or 2. This variation did not appear in the soldier caste, or in *R. santonensis*. The compounds separating the workers of the two seasons, in the three-dimensional component weight (W) space, are on the positive side (fall) methyl 4-tetracosane (m11) and $\times 15$. Negatively, compounds that distinguish the colonies collected in June (summer, 2) are: n-C25 (a14), 9-C23 : 1 (e1), n-C26 (a21), 5Me-C25 (m10), and 9-C23 : 1 (e2).

Multivariate Analysis with Quantities of Cuticular Compounds. A multivariate analysis of the principal components also was performed on the hydrocarbon variables expressed, not in relative proportions, but in quantities (ng/mg of insect). In *R. flavipes* phenotype B was distinguished from other phenotypes by 21 (22 for the soldiers) compounds. The hydrocarbons involved in this analysis were primarily monoenes.

Biometric Study of Phenotypes. The three measurements obtained for each individual are give in Table 5. In *R. flavipes* it seems that phenotype B is clearly distinguished from the other phenotypes with a value of 3.85. Phenotype A is only slightly distinguished (5.21) from the group comprised of phenotypes C and D (5.49 and 5.60, respectively). This last group was comprised of the most abundant phenotypes. Phenotypes E and F also differed a little from groups C + D (index: 5.85). *R. santonensis* seemed intermediate in measurement to group C + D and to group E + F with an index of 5.67. A t test ($\alpha = 0.05$), for

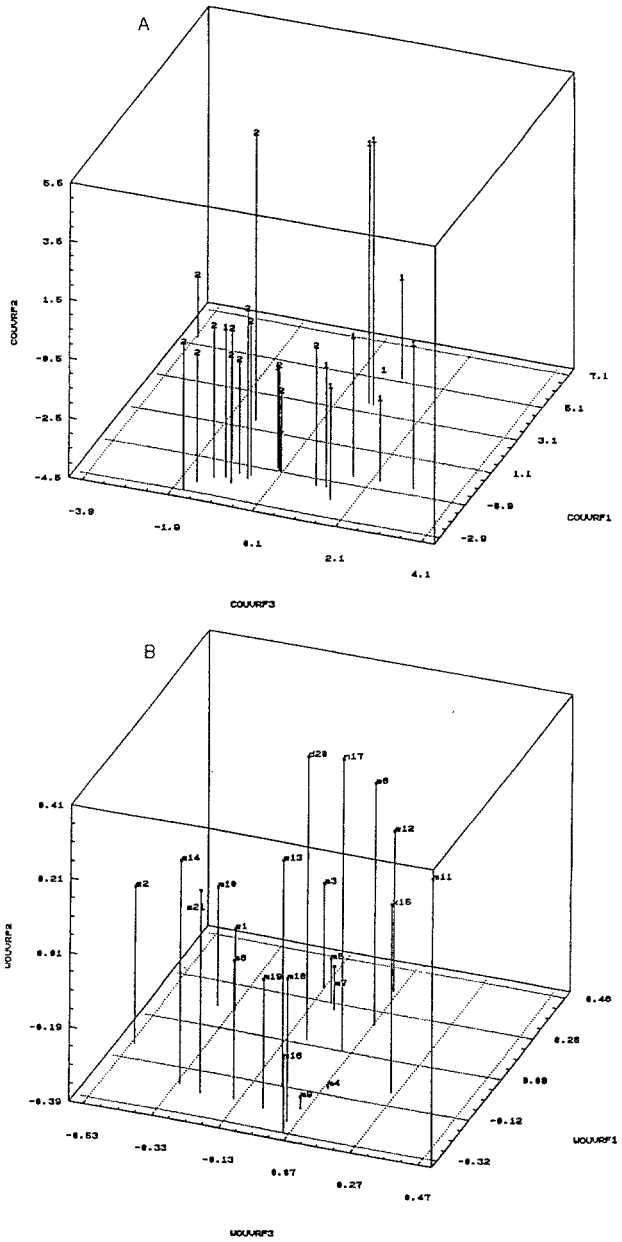


FIG. 11. Plot of seasonal variation of proportions of cuticular hydrocarbons of workers of *Reticulitermes flavipes* after multivariate analysis in principal component (axis *x*: 3, axis *y*: 1, axis *z*: 2). (A) Plot of different colonies (November 1987 (no. 1), June 1988 (no. 2) in principal component space (COUVRF). (B) Plot of different components (21) in component weight space (WOUVRF).

TABLE 5. BIOMETRIC MEASUREMENTS AND INDEX FROM VARIOUS PHENOTYPES AND SPECIES *Reticulitermes*[a]

	[AB]	[BC]	[L]	*N*	*Biometric index*
R. flavipes A	0.55 (0.06)	0.47 (0.08)	5.11 (0.28)	9	5.21
B	0.43 (0.05)	0.46 (0.02)	4.33 (0.09)	8	3.85
C	0.52 (0.08)	0.54 (0.09)	5.18 (0.16)	13	5.49
D	0.53 (0.03)	0.52 (0.08)	5.33 (0.08)	5	5.60
E + F	0.54 (0.05)	0.61 (0.07)	5.09 (0.29)	11	5.85
R.s.	0.60 (0.09)	0.47 (0.09)	5.3 (0.11)	6	5.67
R.m.	0.60 (0.03)	0.51 (0.05)	4.48 (0.13)	5	4.97
R.(l.)g.	0.74 (0.10)	0.55 (0.08)	5.06 (0.17)	6	6.53
R.(l.)b.	0.63 (0.09)	0.51 (0.07)	4.83 (0.24)	5	5.51

[a] A,B,C,D,E + F = phenotypes of *R. flavipes*. R.s. = Reticulitermes santonensis; R.m. = *R. malletei*; R.(l).g. = *R. (lucifugus) grassei*; R.(l).b. = *R. (lucifugus) banyulensis*. AB, BC, and L mean values in millimeters ± Std. Biometric index = (AB + BC) × L. *N* = number of samples.

each distance ([AB], [BC], [L]), was performed, and the results are given in Table 6.

Measurements also were taken on three other species of the genus *Reticulitermes* (*R. malletei, R. (l). grassei*, and *R. (l). banyulensis*). Their indices were very different from those of *flavipes* and *santonensis*, apart from phenotype B, which appeared to be as different as a distinct species. These measurements agreed well with the results on the hydrocarbons and the defensive compounds.

DISCUSSION

This work shows that the cuticular hydrocarbons of *R. santonensis* and *R. flavipes* are qualitatively identical. Their relative proportions are different, but the major and minor constituents are the same. In contrast, the defensive compounds of soldiers are different. Only the quantity of hydrocarbons, (ng/mg of insect) varies from one- to twofold in *R. santonensis*.

On the other hand, a strong chemical polymorphism between sympatric colonies of *R. flavipes* in Georgia appeared in both the hydrocarbons of the

TABLE 6. *t* TESTS OF BIOMETRIC MEASUREMENTS AB, BC, L[a]

	R. flavipes							
	A (9)	B (8)	C (13)	D (5)	E + F (11)	R.s. (6)	R.m. (5)	R.(l.)g. (6)	R.(l.)b. (5)
[AB] *t* test									
R. flavipes	A	4.66 ***	1.04 N.S	0.76 N.S	0.40 N.S	1.19 N.S	2.08 *	4.18 ***	1.78 N.S
	B		3.41 ***	4.21 ***	4.96 ***	4.23 ***	8.06 ***	7.05 ***	4.61 ***
	C			0.37 N.S	0.78 N.S	1.89 N.S	3.23 ***	4.80 ***	2.43 *
	D				0.44 N.S	1.72 N.S	3.20 **	4.74 ***	2.28 *
	E + F					1.51 N.S	2.97 **	4.60 ***	2.09 *
	R.s.						0.00 N.S	2.55 *	0.55 N.S
	R.m.							3.26 **	0.71 N.S
	R.(l.)g.								1.92 N.S
[BC] *t* test									
R. flavipes	A	0.36 N.S	1.92 N.S	0.94 N.S	4.12 ***	0.00 N.S	1.15 N.S	1.90 N.S	0.97 N.S
	B		3.08 **	1.28 N.S	6.74 ***	0.27 N.S	2.13 *	2.69 *	1.56 N.S
	C			0.38 N.S	2.14 *	1.58 N.S	0.90 N.S.	0.24 N.S	0.75 N.S
	D				1.77 N.S	0.85 N.S	0.19 N.S	0.53 N.S	0.18 N.S
	E + F					3.30 ***	3.25 ***	1.54 N.S	2.65 **
	R.s.						0.93 N.S	1.63 N.S	0.83 N.S
	R.m.							1.01 N.S	0.00 N.S
	R.(l.)g.								0.88 N.S

TABLE 6. Continued

	R. flavipes							
	A (9)	B (8)	C (13)	D (5)	E + F (11)	R.s. (6)	R.m. (5)	R.(l.)g. (6)	R.(l.)b. (5)
[L] *t* test									
R.		7.91	0.68	2.11	0.16	1.83	5.72	0.43	1.97
	A	***	N.S	*	N.S	N.S	***	N.S	*
	B		15.57	17.83	8.17	17.62	2.26	9.56	4.46
f			***	***	***	***	*	***	***
l									
a	C			2.34	0.92	1.90	9.57	1.46	3.01
v				*	N.S	N.S	***	N.S	**
i									
p	D				2.43	0.47	11.45	3.24	4.28
e					*	N.S	***	**	***e
s									
	E +					2.14	5.80	0.27	1.88
F						*	***	N.S	N.S
R.s.							11.16	2.90	4.04
							***	**	***
R.m.								6.40	2.87
								***	*
R.(l.)g.									1.80
									N.S

[a] *t* test ($\alpha = 0{,}05$):N.S. = difference no significant, $*P < 95\%$, $**P < 99\%$, $***P < 99.9\%$.

workers and soldiers and in the defensive compounds of the soldiers. This polymorphism can be added to that described by Feytaud (1924), who noted a large morphological polymorphism in *R. flavipes*. Thus, soldiers of *R. flavipes* exhibit six distinct phenotypes with respect to their defensive compounds (A–F), and three phenotypes with respect to cuticular compounds associated with the six phenotypes of defensive compounds associated with those of nestmate workers [A, B, and (CDEF)]. It is important to note that the phenotypes with respect to both cuticular hydrocarbons of workers and soldiers and soldier defensive compounds tightly form a total of three groups in *R. flavipes* [A, B, (CDEF)], of which one is subdivided into four subgroups.

The biometric study of the different phenotypes essentially separated phenotype B of *R. flavipes* from the others. This phenotype is characterized in the soldiers by the presence of two terpenes, γ-cadinene and cadinene aldehyde, and by the relative characteristics of the cuticular hydrocarbons (workers and soldiers), and is close to *R. santonensis* and to phenotype A. In no cases are the variation seasonal or geographic. Haverty et al. (1988) also describe strong

intercolonial polymorphism within species of the genus *Zootermopsis* in the United States.

What might be the role of this hydrocarbon polymorphism? Among the social insects, variability in the proportions of the hydrocarbons could provide a chemical signature characteristic of the colony. Indeed, in these insects, colonies are either open (exchange of individuals between colonies) or closed (no exchange among nonnestmate conspecifics). Recognition of nestmate or nonnestmate is essentially achieved by contact.

Bonavita et al. (1987) and Morel et al. (1988) have shown in the ants *Camponotus vagus* and *C. floridanus* that the cuticular hydrocarbons vary in their relative proportions and that this mixture is used as a nestmate recognition cue. In termites, Howard et al. (1982) and Clément et al. (1985) hypothesized that these substances could serve as markers and that a specific mixture could be responsible for intra- and interspecific aggression.

In the case of the termites *R. santonensis* and *R. flavipes*, their colonies are all of the open type (Clément, 1978, 1980, 1981, also Bagnères, unpublished). That is, there is never any aggression between different colonies of the two species. Experimental work shows that there is no aggression between colonies of the two allopatric species. (*R. santonensis* and *R. flavipes*) (unpublished data). It thus seems that for these two species the variability in proportions does not play a role in communication under the experimental conditions tested in the case of the intraspecific recognition.

These two species, in addition to lacking interspecific aggression between them, have the same type of aggresive behavior toward other species of *Reticulitermes*. Two sympatric colonies in France, one of *santonensis* and one of *grassei*, are very aggressive towards a colony of *flavipes*. The results are the same when colonies of *R. banyulensis* are placed in contact with those of *R. santonensis* or *R. flavipes* (unpublished data).

In contrast to the higher termites such as *Nasutitermes* (Moore, 1964; Vrkoc et al., 1978; Prestwich, 1979, 1984), it appears that differences in defensive compounds among species or groups do not affect the aggressivity of these *Reticulitermes*, even though these compounds have been found to be good taxonomic markers (Gush et al., 1985; Goh et al., 1982; Parton et al., 1981; Everaerts et al., 1988).

Since cuticular hydrocarbon polymorphisms do not appear to have a role in intercolonial recognition in *R. santonensis* and the different phenotypes of *R. flavipes*, do the qualitative differences in the cuticular hydrocarbons have taxonomic value? Several authors use chemical variability to describe different species (Jackson and Blomquist, 1976; Howard et al., 1982). Two morphologically similar species were differentiated on the basis of significantly different compounds by Carlson and Service (1980), who used hydrocarbons as specific markers for *Anopheles* mosquitoes. However, often a greater variability in

the cuticular substances has been described between males and females of the same species than between two different species, as for example with species of *Drosophila* (Luyten, 1982; Nelson et al., 1981). Variability of cuticular compounds associated with developmental stage has been described (Bartelt et al., 1986).

Carlson and Langley (1986) and Lockey (1982, 1984) have, in contrast, described similarities in compounds of closely related species; only variabilities in the proportions were noted. Very different groups of insects, such as ants, flies, hemipterans, etc., possess many compounds in common. The work of Bagnères et al. (submitted), on two ant species, *Formica selysi* (Formicinae) and *Manica rubida* (Myrmicinae), has shown that these two distantly related species have, respectively, 40% and 70% of common products, and in mixed colonies 50% each.

Bagnères et al. (1988) have calculated that 40% of the compounds were shared in common by two species of *Reticulitermes: R. (l). grassei* and *R. (l). banyulensis*, but it seems, after all, that each family (ants, termites) and each genus (*Reticulitermes*, *Kalotermes*) of insect possesses a mixture of diagnostic hydrocarbons. Polymorphism in the relative proportions could be attributable to classical Mendelian genetics.

We now consider the taxonomic significance of (1) the chemical similarity between *R. santonensis* and *R. flavipes* and (2) the chemical polymorphism in *R. flavipes*. Two hypotheses can be proposed to account for the chemical polymorphism of the same blend, of workers and soldiers, of the two species. First, the variability of the observed proportions may reflect intraspecific genetic variation, and in this case we are measuring simple genetics or phenotypic polymorphism.

The genetics of the system, or its phenotypic regulation, merits further study in light of the absence of any intermediates in the different phenotypes. Thus, there are no phenotypic intermediates in the defensive compounds of the soldiers, as for example with geranyllinalool and cadinene.

The mixtures of hydrocarbons and defensive compounds are homogeneous in the colony. This leads to the conclusion that there is either an influence of the social environment or absence of genetic mixing and that the colonies are closed in nature. The test of aggression used was therefore not valid in this case because the colonies were experimentally open. The perception of different signals could result in a nonaggressive but repulsive behavior, such as the closing of galleries.

It can be hypothesized also that *R. santonensis* is a population of *R. flavipes* established in Europe, as different authors have already proposed (Feytaud, 1924). It is possible that a colony of *R. flavipes* was introduced one or two centuries ago and expanded along the Atlantic coast of France and was described as *R. santonensis* by Feytaud (1924). In light of the strong poly-

morphism of *R. flavipes*, its plasticity, and its robustness (Buchli, 1958), the introduction of this species is very possible. If true, this would explain the similarity in the cuticular hydrocarbons and the defensive compounds in the two species. Only sesquiterpenes differ between *R. santonensis* and *R. flavipes* in the southeastern United States. This original colony could have come from an unknown American population.

The other hypothesis is that this chemical variability characterizes sibling species that can be grouped in the same super species: *R. flavipes*. Variation in cuticular hydrocarbons could characterize the genotypes, and their similarity could indicate a close phylogenetic relationship. The genotypes of the workers associated with those of the soldiers could determine the different species. Only the sympatric distribution would be apparent. In the field, two different colonies, even though they appear to occupy the same biotope (e.g., the same piece of wood), could remain separate and live in ecologically distinct ecochemical zones. Unknown mechanisms of reproductive isolation could separate them. This lack of interbreding between the forms would explain the absence of intermediate forms.

There could be three sibling species [A, B, (CDEF)], and even four, including *R. santonensis* [A, B, (CDEF), and S], if the differences in defensive compounds are considered nondiagnostic, or even seven different species, if the defensive compounds characterize the genotypes.

In support of this hypothesis, *R. virginicus*, separated from *R. flavipes* and described by Banks and Snyder in 1920, is chemically very close to *flavipes*, sharing many similar hydrocarbons. We even have chromatograms from *R. flavipes* identical to those of certain colonies of *R. virginicus* collected in Georgia, respectively, identified by their sexuals. Moreover, their defensive compounds, described by Zalkow et al. (1981), are also identical.

The cuticular differences noted between *R. virginicus* and *R. flavipes* are of same order as those between *R. (l.) grassei* and *R. (l.) banyulensis*, which belong to the same superspecies *lucifugus* (Clément, 1977). One could speak of a superspecies *R. flavipes* in the United States that also includes *R. virginicus*. They are separated morphologically, differing in wing coloration of the sexuals, but a preliminary biometric and multivariate study group *flavipes* as phenotype B. *R. flavipes* and *R. virginicus* are reproductively isolated (different sex pheromones and dates of mating flights) and by aggression as well, since the workers are mutually aggressive, just as in the case of *R. (l.) grassei* and *R. (l.) banyulensis* in Europe (Clément, 1986).

To decide between these two hypotheses (the second is more likely, in our opinion), the entire range of *R. flavipes* in the United States needs to be studied. Enzymatic polymorphism might allow us to address the genetic questions posed by this species, by distinguishing phenotypes from genotypes and calculating the genetic distance between the different groups. Controlled mating of the sex-

uals of *R. flavipes* as done for *R. (l.) grassei* and *R. (l.) banyulensis* by Clément (1979) and studies of the reproductive mechanisms of the species (mating flights, sex pheromones, fecundity of offspring) also will be needed in order to address the biological definition of this species (Mayr, 1963).

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REPLACEMENT OF CARCINOGENIC SOLVENT HMPA BY DMI IN INSECT SEX PHEROMONE SYNTHESIS

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Abstract—Hexamethylphosphoric triamide (HMPA) is a good solvent for the alkylation of terminal acetylenes; however, it has been proven to be a carcinogen towards rats and should be considered as a serious risk to man when a large-scale synthesis is involved. Thus the search for a safe alternative is important. The physical properties of 1,3-dimethyl-2-imidazolidinone (DMI) are similar to DMPU (1,3-dimethyl-2-oxo-hexahydropyrimidine) and HMPA, but the potential toxicological risk of DMI is less than DMPU and HMPA. When used in the alkylation of terminal acetylenes, DMI is comparable with HMPA, thus it is a good alternative solvent to the carcinogen HMPA in the alkylation because it provides a safer working environment than DMPU and HMPA.

Key Words—Smaller tea tortrix, *Adoxophyes fasciata*, (*Z*)-9-tetradecenyl acetate, (*Z*)-11-tetradecenyl acetate, (*E*)-11-tetradecenyl acetate, 10-methyldodecyl acetate, hexamethylphosphoric triamide, 1,3-dimethyl-2-oxo-hexahydropyrimidine, 1,3-dimethyl-2-imidazolidinone.

INTRODUCTION

Most insect sex attractants have at least one long straight chain ($C_{10}-C_{16}$) alcohol or acetate with a double bond (Ando et al., 1977) and are typically prepared by a Wittig reaction between either an aliphatic or functionalized nonstabilized triphenylphosphonium ylide and either an aliphatic or functionalized aldehyde. They also can be prepared from an acetylenic tetrahydropyranyl ether [THP$-$O$-$(CH$_2$)$_m-$C$\equiv$C$-$(CH$_2$)$_n-$CH$_3$, THP = tetrahydropyran-2-yl]

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(Henrick, 1977) by selective reduction. These intermediates are prepared by the alkylation of terminal acetylenes with alkyl halides. For example, (*Z*)-9-tetradecenyl acetate (Z9-TDA) and (*Z*)-11-tetradecenyl acetate (Z11-TDA) are sex pheromones of the smaller tea tortrix *Adoxophyes fasciata* Walsingham (also a serious tea pest in the northern part of Taiwan) and were generally synthesized by a Wittig reaction (Tamaki et al., 1971).

However, the Wittig reaction is usually not as stereoselective as the alkylation route (Henrick, 1977), and our previous experiments also indicated that the Wittig reaction can (under certain conditions) lead to a *cis–trans* mixture in a 5:1 ratio (Lo et al., 1988). Further purification by liquid chromatography on silica gel impregnated with 20% silver nitrate could afford pure compound (95% active isomer) in low yield (less than 10%). Alkylation followed by partial selective catalytic hydrogenation over Lindlar catalyst generally gave high yields of the corresponding *Z* olefin (Henrick, 1977); therefore, the acetylenic route was considered, but the yield and the time required for the alkylation varied with reagents and solvent systems. For example, the classical alkylation procedure carried out in liquid ammonia gave a yield of 56.9%, with a reflux time of 4–5 hr (Green et al., 1967), but this reaction was affected by the solubilities of alkyl halides and metal 1-alkyn-1-ide in liquid ammonia and was not successful with increased length of the $(CH_2)_n$ chain (Warthen, 1968; Warthen and Jacobson, 1968; Kochansky et al., 1975). The combination of HMPA with tetrahydrofuran (THF) provided a good alternative (Schwarz and Waters, 1972; Ando et al., 1977), and the alkylation was worked up within 30 min with a yield of 85–92%. However, a long-term inhalation toxicity study with rats indicated that cancer emerged at levels as low as 400 ppb after eight months, and 50 ppb after 13 months, and a permissible airborne exposure limit of 0.5 ppb was suggested (Schmutz, 1978). Consequently, HMPA must be considered as a serious risk to man and should not be used when large-scale synthesis is considered. Thus the search for a safe substitute for HMPA is necessary.

The cyclic urea DMPU was reported to exhibit the same effects as HMPA in oxirane opening with lithium acetylide, in a Wittig olefination, in the double deprotonation of a nitroalkane, in the Michael addition of lithiodithiane to cyclohexenone, and in selective generation of certain enolates (Mukhopadhyay and Seebach, 1982). DMPU also can replace HMPA in stereoselective alkylations of chiral α-nitro keto imine dianions (Denmark and Ares, 1988). The possible usage of DMPU in insect sex pheromone synthesis was not reported until 1988, and it proved to be a better solvent then HMPA in the coupling reaction of an alkyne, or a functionalized alkyne, with an alkyl halide (Bengtsson and Liljefors, 1988). However, DMPU is a mutagen in the *Drosophila* SLRL (sex-linked recessive lethal) test (Zijlstra and Vogel, 1988), indicating a potential toxicological risk to man.

DMI is an aprotic basic solvent, and the physical properties are similar to

HMPA (Table 1) (Normant, 1967; Barker et al., 1979). A preliminary *Salmonella* mutagenic test based on the revised method of Maron and Ames (1983) showed a negative response (You, 1988).

A comparison of the mutagenic assay system was made of 439 references on selected carcinogens and noncarcinogens (Brown et al., 1979). Of the compounds reviewed, 19 (90%) of the 21 carcinogens and 3 (50%) of the six noncarcinogens were active in the *Drosophila* system, whereas 37 (84%) of the 44 carcinogens and 2 (25%) of the eight noncarcinogens were active in the *Salmonella* system; thus, the potential carcinogenic risk of DMPU should be noted. Furthermore, the actue toxicity (CNS toxicity) of DMI (LD_{50} = 2840 mg/kg, mice) was lower than DMPU (LD_{50} = 1300 mg/kg, mice) (Lien and Kumler, 1968). Thus the use of DMI should be considered safer than the use of DMPU on the basis of available toxicity data (Table 1).

Seebach et al. (1982) concluded that DMPU will become generally more useful than DMI, because a heavy precipitate was observed below ca. −30°C from a 2 : 1 mixture of THF and DMI, while a 2 : 1 mixture of THF and DMPU was still clear and homogeneous at −78°C. The same is noted by Mukhopadhyay and Seebach (1982): they found that a 1 : 2 mixture of DMPU with THF was clear and homogeneous down to −90°C.

However, alkylation of an 1-alkyn-1-ide ion with a primary alkyl halide

TABLE 1. PHYSICAL AND TOXICOLOGICAL PROPERTIES OF DMI, DMPU, AND HMPA

	DMI	DMPU	HMPA
bp (°C/torr)	220/754	230/754	235/760
mp (°C)	8.2[a]	< −20	7.20
Density (g/ml) at 25°C	1.0519	1.0596	1.0253
Viscosity (cP) at 25°C	1.944	2.934	3.5cS (60°C)[b]
Dielectric constant at 25°C	37.60	36.12	30 (20°C)
Dipole moment (Debye)	4.09	4.23	4.31-5.37 (25°C)
Toxicity			
Acute (mice, mg/kg)	2840	1300	NA[c]
Mutagenic			
Salmonella system	−	NA	NA
Drosophila system	NA	+	NA
Carcinogenic (rat)	NA	NA	+

[a] Laboratory studies showed that DMI (>99%, Tokyo Kasei Kogyo Co., Ltd.) was crystallized in refrigerator at −10°C, and started to melt at −4°C, indicating the mp should be lower than −4°C.

[b] Laboratory studies using a Brookfield digital viscometer indicated the viscosity of HMPA was 4.8 cP at 19°C.

[c] Not available.

usually can be carried out at temperature of 0–25°C, and the yields are usually high (Schwarz and Waters, 1972; Brattesani and Heathcock, 1973; Henrick, 1977; Bengtsson and Liljefors, 1988; Brandsma, 1988). At this temperature range, DMI will be a clear and homogeneous solvent (Table 1) and will be a better choice than DMPU when safety is considered.

The use of DMI as a substitute solvent for HMPA has been reported only in the preparation of trimethylsilylsodium and trimethylsilylpotassium (Sakurai and Kondo, 1975), in the reaction of a trialkylchlorosilane with lithium (Sakurai and Kondo, 1976), in the preparation of homoallylic alcohol derivatives (Mukaiyama et al., 1980), in the dehydration and dehydrohalogenation of saturated and unsaturated alcohols and dienyl halides (Spangler et al., 1981), and in Ullmann ether synthesis (Oi et al., 1988). There is no report specifically on use of DMI in pheromone synthesis. Therefore, we report here the alternative alkylation route in the syntheses of insect sex pheromones of Z9-TDA and Z11-TDA of the smaller tea tortrix, and the first use of DMI instead of DMPU and HMPA during the organometallic reaction in the syntheses of sex pheromones.

METHODS AND MATERIALS

DMI (>99%) was purchased from Tokyo Kasei Kogyo Co., Ltd., and was stored in serum-capped bottles under nitrogen over 4 Å molecular sieves.

Gas–liquid chromatography (GLC) analysis was performed on the synthetic materials with an Hewlett Packard 5890 equipped with a flame ionization detector (FID). A glass column of 3% OV-1 on 80–100 Chromosorb W HP, 2 mm × 1.8 m was used for analysis of the synthetic intermediates with the initial oven temperature at 160°C (1 min), then programmed to 240°C (8°C/min). A glass column of OV-275 on 80–100 Chromosorb W AW was used to analyze the final synthetic pheromones; the initial oven temperature was 150°C (1 min), then programmed to 220°C (4°C/min). Nitrogen was used as carrier gas at the rate of 29.9 ml/min, and air and hydrogen flows were set as recommended for FID.

GC-MS measurements were recorded on an Hewlett-Packard 5890A GLC combined with a 5970B mass selective detector (EI, 70 eV). Infrared data were obtained with a Perkin Elmer 567 infrared spectrophotometer, and ^{1}H nuclear magnetic resonance data were obtained with a Varian VXR-300 spectrometer.

14-(2-Tetrahydropyranyloxy)-3-tetradecyne (IIa), and 14-(2-Tetrahydropyranyloxy)-5-tetradecyne (IIb). To a stirred solution of the ω-tetrahydropyranyloxy-l-alkyne (Ia, or Ib, 23 mmol) in dry solvent A (23 ml, Table 2) at 0°C under nitrogen atmosphere, *n*-butyllithium (*n*-BuLi, 1.6 M in hexane, 21.5 ml, 34.4 mmol) was added dropwise. The reaction mixture was stirred for 15 min. The alkyl bromide (34.5 mmol) in dry solvent B (40 ml, Table 2) was added

TABLE 2. PREPARATION OF YNYL TETRAHYDROPYRANYL ETHERS (IIa, IIb)[a]

Solvent[b]		Yield (%) in 2 hr[c]	
A	B	IIa	IIb
THF	THF	8	8
HMPA	HMPA	78	88
DMI	DMI	83	90
THF	HMPA	78	75
THF	DMI	88	75

[a] Compounds were purified with $AgNo_3$ solution, and GLC analyses and consistent spectra were obtained for all compounds.
[b] ω-Tetrahydropranyloxy-1-alkyne (Ia and Ib) was added in dry solvent A, and alkyl bromide was added in dry solvent B.
[c] Based on 1-alkynyl tetrahydropyran-2-yl ether (IIa and IIb) starting materials.

slowly. The resulting solution was stirred for 15 min at 0°C, and then stirred for an additional 2 hr at room temperature. The reaction mixture was worked up within 30 min by pouring into 200 ml ice water and extraction with hexane (3 × 200 ml). The combined organic fractions were washed with water, brine, and dried ($MgSO_4$). The solvent was removed using a rotary evaporator to afford the crude ynyl tetrahydropyranyl ethers (IIa, IIb) (Figure 1).

Subsequent purification by treating with 1.7% silver nitrate solution (in ethyl alcohol) was applied to remove the unreacted terminal alkynes (Ia, Ib). The unreacted terminal alkyne formed a white precipitate with silver nitrate, while the yellow ynyl tetrahydropyanyl ether (IIa, IIb) remained in solution and could be collected by filtration. The white precipitate (metal acetylide) could explode when dry, therefore, the precipitate was treated with 65% nitric acid solution to recover the unreacted tetrahydropyranyl ethers (Ia, Ib). The purification was unnecessary when the yield was higher than 90%.

$$\underset{\text{Ia, b}}{\text{THP}-\text{O}-(\text{CH}_2)_m-\text{C}\equiv\text{CH}} \xrightarrow{1.7\%\ \text{AgNO}_3} \underset{\text{(precipitated)}}{\text{THP}-\text{O}-(\text{CH}_2)_m-\text{C}\equiv\text{CAg}}$$

$$\xrightarrow{65\%\ \text{HNO}_3} \underset{\text{(recovered)}}{\text{THP}-\text{O}-(\text{CH}_2)_m-\text{C}\equiv\text{CH}} + \underset{\text{(precipitated)}}{\text{AgNO}_3}$$

(Z)-11-Tetradecen-1-ol (IVa), and (Z)-9-Tetradecen-1-ol (IVb). The tetrahydropyranyl ether IIa or IIb (18.7 mmol) was converted to the corresponding alcohol by treatment for 3 hr with *p*-toluenesulfonic acid (*p*-TsOH, 10.6 g) in refluxing methanol (300 ml) in yields of 90% for IIIa, and 92% for IIIb.

Subsequent partial hydrogenation followed, and a solution of the IIIa or

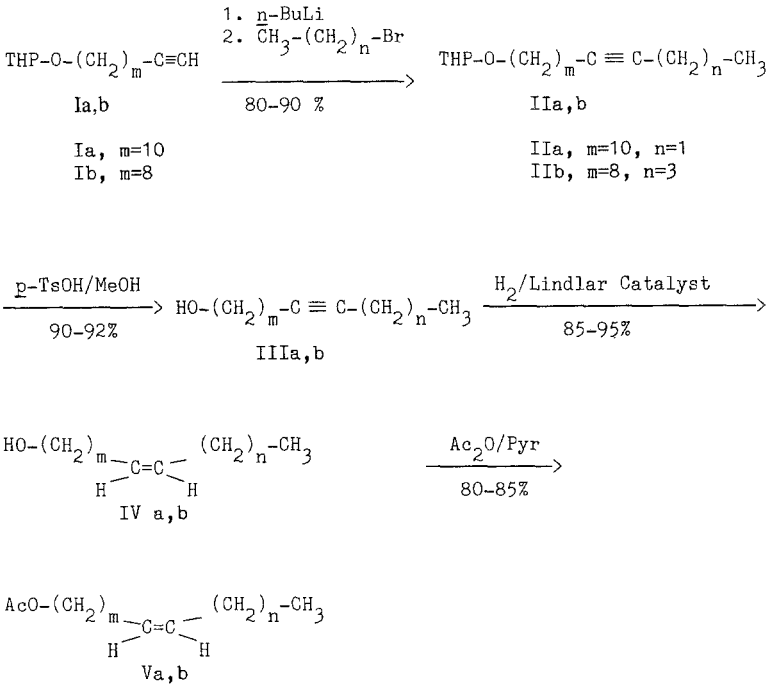


FIG. 1. Synthesis of (*Z*)-11-tetradecen-1-yl acetate (Va) and (*Z*)-9-tetradecen-1-yl acetate (Vb).

IIIb (16.8 mmol) in methanol (100 ml) was hydrogenated at room temperature using a Lindlar catalyst (1.4 g) and quinoline (0.9 ml). When the required amount of H_2 for one double bond had been absorbed (403 ml at 760 mm), the reaction was stopped. The catalyst was removed by filtration, the methanol was evaporated at reduced pressure, and the residue was dissolved in ether. The ether solution was washed with 5% HCl, sat. aq. $NaHCO_3$, water, and brine. The ether solution was dried ($MgSO_4$) and concentrated in a rotary evaporator to give products IVa or IVb in yields of 85% or 95%, respectively.

(Z)-11-Tetradecen-1-yl acetate (Va), and (Z)-9-Tetradecen-1-yl acetate (Vb). A solution of IVa or IVb (14.8 mmol), acetic anhydride (Ac_2O, 14 ml), and pyridine (Pyr, 12ml) was refluxed overnight, then poured into ice water and neutralized with sat. aq. NaOH. The aqueous phase was extracted with *n*-hexane and dried ($MgSO_4$). The solvent was removed under vacumn, and the residue was distilled to give colorless oily pheromone products of Va or Vb. The physical data for these pheromones were as followed.

Va: bp 71.5–73.0°C/0.0075 torr (uncorrected); yield 80% (based on IVa); purity 95%; MS(EI): *m/e*(%) 194 (M^{+}·-AcOH, 8), 43(100); IR(KBr) 3008,

2928, 2856, 1744(Co), 1654, 1464, 1366, 1238 (primary acetate), 1040, 720 (*cis* HC=CH); NMR($CDCl_3$/TMS): 0.95(t, 3H, CH_3), 1.28[s, 22H, $(CH_2)11$], 2.04(s, 3H, CH_3CCOO), 4.05(t, 2H, CH_2OAc), 5.30–5.36 (m, 2H, HC=CH).

Vb: bp 75.2–75.4°C/0.0075 torr (uncorrected); yield 85% (based on IVb); purity 94%; MS(EI): *m/e*(%) 194($M^{+\cdot}$-AcOH, 7), 43(100); IR(KBr) 3012, 2928, 2860, 1744(Co), 1656, 1468, 1366, 1240(primary acetate), 1040, 722 (*cis* HC=CH); NMR($CDCl_3$/TMS): 0.89(t, 3H, CH_3), 1.30[s, 22H, $(CH_2)11$], 2.04(s, 3H, CH_3CCOO), 4.05(t, 2H, CH_2OAc), 5.32–5.37(m, 2H, HC=CH).

Field Tests. Field testing was carried out in tea plantations in the Wenshan Substation of the Taiwan Tea Experiment Station, from July 11 to August 8, 1989. The synthetic pheromones (0.01 mg) loaded in polyethylene tubes were *Z*9-TDA, *Z*11-TDA, *E*11-TDA, which was isomerized from *Z*11-TDA by aqueous $NaNO_2$ and HNO_3(Sonnet, 1974), and 10-methyldodecyl acetate (unpublished result) in a ratio of 63 : 31 : 4 : 2 (Tamaki et al., 1979). Dispensers were mounted at the center of the sticky wing traps.

RESULTS AND DISCUSSION

The alkylation in the DMI solvent systems was almost complete within 2 hr, and the yield was a little better than the alkylation in the HMPA system, whereas the yield of alkylation in the THF system was very low, indicating that alkyl bromides essentially do not react with lithium acetylide in THF. The yield also was decreased when THF was used as a cosolvent of HMPA or DMI (Table 2); the low yield indicated that a good alkylation yield depends on the substrate structure and solvent polarity.

The good results with DMI indicated that the preparation of sex pheromones by alkylation followed by hydrogenation not only gave good isomeric purities, but also proved DMI could be a safe alternative solvent to HMPA in insect sex pheromone synthesis, especially when large-scale synthesis was considered.

The total moth catch in the tea plantation was low unless the proportion of each component was varied (S.N. Hsiao, 1989, unpublished results, Wenshan Substation of the Taiwan Tea Experiment Station. In one of her field tests, she found that the total moth catch could be increased significantly by varying the proportion of each component to 47 : 50 : 1 : 2), indicating that the best pheromone formulation for the smaller tea tortrix was not established in Taiwan, and further research on the pheromone proportion of 14 : 6 : 1 : 2 (Tamaki et al., 1979) or other possible proportion combinations should be considered.

Our report has led to the conclusion that if a reaction could be conducted at a temperature higher than 0°C, use of DMI should be considered. We also believe that DMI will become a more favored solvent than DMPU and HMPA in the future.

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OLFACTORY RESPONSE OF EASTERN SPRUCE BUDWORM LARVAE TO RED SPRUCE NEEDLES EXPOSED TO ACID RAIN AND ELEVATED LEVELS OF OZONE[1]

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Abstract—Second-instar eastern spruce budworm larvae, *Choristoneura fumiferana* (Clem.), were tested in a two-choice, Y-type, wind-tunnel olfactometer for preferences for red spruce, *Picea rubens* Sarg., needles exposed for eight weeks to charcoal-filtered air (CFA) or 0.15 ppm ozone (O_3) in combination with acidified rainfall at pH 4.2 or 3.0. Volatiles from needles treated with CFA plus pH 4.2 rain (control) were preferred over those from needles exposed to pH 3.0 rain + O_3 or O_3 alone; O_3-treated needles were chosen over those exposed to pH 3.0 rain + O_3. No preference was shown between the pH 4.2 and 3.0 rain treatments. Larvae chose purified air flowing through the olfactometer in preference to needle volatiles from the pH 3.0 rain + O_3 treatment.

Key Words—Eastern spruce budworm, *Choristoneura fumiferana*, Lepidoptera, Tortricidae, red spruce, *Picea rubens*, atmospheric deposition, acid rain, ozone, host volatiles, larval olfaction.

INTRODUCTION

The eastern spruce budworm, *Choristoneura fumiferana* Clem. (Lepidoptera: Tortricidae), feeds on several coniferous species of the spruce-fir forest com-

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plex in eastern North America. One of its hosts is red spruce, *Picea rubens* Sarg.

In recent years there has been considerable interest in the apparent decline or deterioration of red spruce in high-elevation forests of the Appalachian Mountains (Woodman, 1987). Although numerous theories have been put forth to explain this phenomenon, it has been suggested that the cumulative effects of multiple stress factors including air pollution and acidic precipitation are involved (Siccama et al., 1982). While the northeastern mountains are remote from sources of industrial pollutants, they receive high rates of acid deposition in the cloud-covered high-elevation forests (Johnson and Siccama, 1983). According to Woodman (1987), "Ozone (O_3) is the only regionally dispersed gaseous pollutant known to have injured foliage, decreased resistance to insects and disease, and increased mortality of sensitive tree species over a wide geographic range."

High-elevation forest ecosystems are highly complex and may be impacted by acid deposition and gaseous pollutants through various biological pathways. Little is known about the possible effects of pollutants on the production and emission of host-plant volatiles, or if alterations could be detected by an associated insect herbivore.

Ascoli and Albert (1985) reported that the second-instar eastern spruce budworm larva can detect host-plant foliage volatiles and discriminate between odors of different hosts. The purpose of this investigation was to determine if the attraction of second-instar larvae to red spruce needles was affected by laboratory exposure of seedlings to elevated levels of O_3 and/or acidified rain.

METHODS AND MATERIALS

Cultural Practices. Two-year-old red spruce seedlings, grown from a common seed source at the USDA Forest Service, Northeastern Forest Experiment Station, Durham, New Hampshire, were transplanted outdoors in September in 10.2 × 10.2 × 35.6-cm plastic pots containing equal parts (v/v) of peat moss and vermiculite. Each cubic meter of medium was amended with 3 kg lime, 1.2 kg superphosphate (0–20–0), 4.5 kg Osmocote (18–12–6) slow-release fertilizer, 0.9 kg Aqua-Gro nonionic soil wetting agent, and 0.04 kg Peter's trace elements.

In early December, seedlings were placed in cold storage at 5°C. Dormant plants were removed from cold storage in January and 100 seedlings of uniform size transferred to 1.07-m-diameter continuous stirred tank reactor (CSTR) chambers (Heck et al., 1978) located in a controlled temperature room. Illumination (15 hr photoperiod, 0400–1900 EST) was provided to each chamber by a metal halide lamp (MS1000/BU, Phillip's Lighting Corp., Bloomfield, New Jersey) that emitted 280 W/m^2 photosynthetic active radiation as measured

with a LI-COR photometer (model 85B; LI-COR, Lincoln, Nebraska) at the top of the plants. Chamber temperature was maintained at 24 ± 1°C during the light period and 20 ± 1°C during the dark period. Relative humidity ranged from 36 to 42%. All seedlings were watered twice weekly with 250 ml tap water.

Pollutant Treatments and Monitoring. Starting at the end of January, at budbreak, 20 seedlings each were subjected to one of the following treatments: (1) charcoal-filtered air plus pH 4.2, acidified rain (control), (2) charcoal-filtered air plus pH 3.0 acidified rain (AR treatment), (3) control plus 0.15 ppm O_3 (O_3 treatment), or (4) AR treatment plus 0.15 ppm O_3 (AR + O_3 treatment).

Ozone fumigations began daily, seven days each week, at 1000 hr EST and lasted for 6 hr. Ozone was generated by a corona discharge generator (Ozone Research and Equipment Co., model 03V10-0, Phoenix, Arizona). Ozone concentrations were monitored (Thermal Electron Corp., model 49 Ozone Analyzer, Hopkinton, Massachusetts) just above the foliage in the center of each CSTR chamber with an automated time-sharing system. The monitor was calibrated daily with a Monitor Labs model 3500 Permacal O_3 calibrator (Monitor Labs, San Diego, California).

Acidified rain (AR) treatments were applied at 0700 hr three times weekly (Monday, Wednesday, Friday) by dispensing 1.25 cm rain (approx. 10 min) through a nozzle (Bete Fog Nozzle P80, Greenfield, Massachusetts) in each CSTR chamber designated to receive rain treatment. This allowed sufficient time for needle surface to dry before the initiation of O_3 fumigation at 1000 hr. Each nozzle was positioned in the center of the chamber just below the impeller. Impeller action and nozzle spray pattern combined to give uniform rain dispersion. The distribution of rain droplet size, measured using a water-sensitive paper method (Ciba-Geigy, 1981), was: 58% 82–165 μm, 30% 166–260 μm, and 12% 261–345 μm. Rain treatments were prepared for each precipitation event by adjusting the pH of deionized water to either pH 4.2 or pH 3.0 with a mixture of two parts 1 M H_2SO_4 and one part 1 M HNO_3.

The O_3 and rain treatments were continued for eight weeks from late January until early April. First-year needles were then plucked at random with fine forceps from the distal 3 cm of terminal branches sampled in the cardinal axes of seedlings from each treatment. Needle samples were sealed immediately in plastic bags, refrigerated at 5°C, and used within 4 hr after removal from the plant (Ascoli and Albert, 1985). Needles were brought to room temperature for 5 min prior to bioassay. Four needles were used as a sample in each test and were replaced each hour.

Bioassay. Hibernating second-instar spruce budworm larvae were obtained from the Insect Production Unit, Forest Pest Management Institute, Canadian Forestry Service, Sault Ste. Marie, Ontario. They were maintained on gauze at 5°C without food and water. Prior to bioassay, a section of larvae-containing

gauze was placed in a covered 400-ml beaker at 25°C. After 24 hr, the active larvae that crawled up the side of the beaker were selected and used only once for bioassay.

The two-choice, wind-tunnel olfactometer system used was that developed by Ascoli and Albert (1985). Air was purified by passing through a charcoal filter, a drierite filter, a molecular sieve, and deionized glass-distilled water. The airstream was divided into two equal parts (monitored by flowmeters) and passed over foliage samples into the two branches of the Y-tube olfactometer at a flow rate of 175 ml/min (614 cm/min velocity). Exhaust air was directed to a fume hood.

The Y tubes were tested for bias in preliminary trails; only unbiased tubes were used. Between trials, the position (right and left arms) of the Y tube was switched randomly and the airflow was switched randomly to each arm of the Y tube.

Each trial began when a larva was placed on the wire within the Y tube, ca. 1 cm from the bottom, and ended when the larva was at least 1 cm past the junction of the Y. Only those trials were recorded in which the behavior exhibited by the larvae was a persistent crawl up the tube followed by a rapid decision at the junction of the Y. Other behaviors (Ascoli and Albert, 1985) were ignored. Ambient temperature ranged from 22 to 25°C.

Larval response to needle samples from each of the pollutant treatments was compared in the two-choice bioassay system using all possible spruce needle–treatment combinations. Each treatment also was compared with only purified air supplied to the olfactometer. Twenty trials were made for each comparison. Test results were analyzed by Yates' chi-square test with the null hypothesis that the airstream choice by a larva was random ($P = 0.5$) (Sokal and Rohlf, 1969).

RESULTS AND DISCUSSION

Second-instar eastern spruce budworm larvae exhibited a preference for red spruce needle volatiles (control) over purified air supplied to the olfactometer (no needles) alone (Table 1). The AR treatment did not appreciably affect this choice. Volatiles from needles treated with O_3 were chosen less often than those from the control needles. None of the larvae tested chose the AR + O_3-treated needles over the control.

The combination of AR + O_3 resulted in needle volatiles that were chosen less often by a significant number of larvae than volatiles from needles exposed to the AR or O_3 treatments alone. Larvae avoided volatiles from needles treated with AR + O_3 by a 3 : 1 margin over larvae that chose the purified air supplied to the olfactometer.

TABLE 1. RESULTS OF TWO-CHOICE OLFACTOMETER TRIALS OF PREFERENCES OF SECOND-INSTAR *Choristoneura fumiferana* LARVAE FOR *Picea rubens* NEEDLES EXPOSED TO ELEVATED LEVELS OF OZONE AND/OR ACIDIFIED RAIN

Choice			Frequency			
A	vs.	B	A	B	χ^2	$P > \chi^2$
Control[a]	vs.	air[b]	16	4	6.05	0.014
	vs.	AR	11	9	0.05	0.823
	vs.	O_3	15	5	4.05	0.044
	vs.	AR + O_3	20	0	18.05	0.000
AR	vs.	air	14	6	2.45	0.118
	vs.	O_3	16	4	6.05	0.014
	vs.	AR + O_3	16	4	6.05	0.014
O_3	vs.	air	12	8	0.45	0.502
	vs.	AR + O_3	17	3	8.45	0.004
AR + O_3	vs.	air	5	15	4.05	0.044

[a] Treatments applied to *P. rubens* seedlings: control, charcoal-filtered air plus pH 4.2 acidified rain; AR, charcoal-filtered air plus pH 3.0 acidified rain; O_3, control plus 0.15 ppm O_3; AR + O_3, AR plus 0.15 ppm O_3.
[b] Purified air supplied to the olfactometer.

The response of larvae to AR-treated needles was not significantly different from that to the control needles. Needles treated with AR were preferred in a significantly greater number of trials to needles exposed to O_3 alone.

To summarize, the following hierarchy of treatments was revealed from choices made by the budworm larvae: control > AR + O_3, but = AR; AR > O_3, and > AR + O_3; O_3 > AR + O_3. The AR + O_3 treatment produced changes in needle volatiles that resulted in significant numbers of larvae selecting control > O_3 > AR > air over this treatment. What changes occurred in the needle volatiles is subject to speculation. A study of the evolution of volatiles in red spruce under pollution stress may help clarify this question.

There is evidence that many plant volatiles may be affected by pollutants (Bucher, 1984). Ethylene evolution, for example, is correlated with the O_3 dose in a large number of plant species (Tingey et al., 1976). Acid rain itself may induce foliar membrane damage (Chia et al., 1984).

The reason needles that received the AR + O_3 treatment produced such a nonattractive response by budworm larvae may include needle cuticular structure as well as physiological processes. The needle cuticle itself probably represents a barrier to volatile emissions (Hanover, 1972). The release of volatiles is normally via the stomatal pathway. Red spruce needles exposed to the AR

+ O_3 treatment exhibited severe epistomatal wax injury expressed as agglomerated wax tubules and sheets of wax; O_3 alone did not produce this effect (Krause and Cannon, 1991). In addition to altered wax structure, reports in the literature indicate that both O_3 and acid rain may cause at least partial stomatal closure (Neufeld et al., 1985; Jensen and Roberts, 1986; Tseng et al., 1988). A recent study of xylem water potential of red spruce seedlings exposed to O_3 and acid rain provides evidence that exposure to these pollutants may produce a more favorable balance of plant moisture by influencing transpirational water loss via stomatal closure (Roberts and Cannon, 1989).

The combined effects of stomatal closure with epistomatal injury could have altered the evolution of needle volatiles. Detection of altered evolution by budworm larvae caused the rejection of the AR + O_3-treated needles. Little is known, however, about this response and the plant physiological responses to these pollutants that would impact the evolution of plant volatiles. More information is needed about how atmospheric deposition influences evolution of plant volatiles and subsequent response by eastern spruce budworm.

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A SIMPLE METHOD FOR ANALYSIS OF INSECT CUTICULAR HYDROCARBONS

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Abstract—Cuticular hydrocarbons from tiny pieces of insect cuticle can be analyzed directly by gas chromatography without solvent. The cuticle, sealed in a glass capillary, is introduced into the heated injector area of a gas chromatograph, and the glass capillary crushed after heating for 4 min. Linked gas chromatography–mass spectrometry can be performed on selected parts of individual insects, such as an antenna, leg, or abdominal sclerite, to give qualitative and quantitative analysis of the substances on a small area of the body.

Key Words—Cuticular hydrocarbons, insect cuticle, chemotaxonomy, gas chromatography.

INTRODUCTION

Interest in the cuticular lipids of insects has grown steadily in recent years, encouraged partly by the discovery that these substances play a part in species recognition (Howard et al., 1982; Bonavita-Cougourdan et al., 1987) but also by the discovery that they can be used as a chemotaxonomic tool (Carlson and Service, 1980; Lockey, 1982, 1984; Hadley et al., 1981; Haverty et al., 1988).

Preparation of samples for gas chromatographic analysis of hydrocarbons is usually made by solvent extraction of whole bodies with pentane, hexane, dichloromethane, or chloroform. We have been concerned that this process may contaminate the cuticle sample with other materials, such as from exocrine glands. The Dufour gland of ants is a known source of hydrocarbons and related

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compounds. We have noted one recent report of cuticular extracts of an ant, *Pseudomyrmex ferruginea* (Mintzer et al. 1987), where it is highly probable some of the extracts also contained a portion of the contents of the Dufour glands (Attygalle et al., 1990). We have shown that the postpharyngeal gland in the head of ants is a source of hydrocarbons very similar to the cuticular hydrocarbons (Bagnères and Morgan, 1990). Contents of many other glands, for example, the mandibular glands of bees or the pygidial glands of beetles may be accidentally added to the hydrocarbon extract of cuticle. Such extraneous substances may lead to inconsistent results and erroneous conclusions. In addition, since rather large samples and several manipulations are required to make the necessary extracts, the process is not applicable to single insects.

Brill and Bertsch (1985) recognized and listed a number of disadvantages of solvent extraction for analysis of cuticular hydrocarbons. They used a pyrolysis unit in the gas chromatograph injection. They heated a whole insect rapidly to 300°C for 5 sec in the pyrolysis unit and showed that this produced a higher yield of hydrocarbons than solvent extraction. Their method also caused the extraction of materials from inside the body (in their case, including alkaloids from the venom gland), which we particularly wanted to avoid.

We wish to demonstrate here that errors can be avoided, that single insects or separate parts of an insect can be examined for their cuticular lipids by gas chromatography or linked gas chromatography–mass spectrometry, by the solid sampling method we have used for some time (Morgan and Wadhams, 1972). The technique also has been adapted for the study of lepidopteran sex pheromones (Attygalle et al., 1987). Sensitive and accurate analysis can be performed on tiny fragments of cuticle, so that a complete spectrum of volatile (C_1 to around C_{15}) and nonvolatile (C_{15} to C_{45}) cuticular compounds can be obtained.

METHODS AND MATERIALS

Live insect material was obtained from various sources and maintained in the laboratory until ready for dissection. Three species of ant (Hymenoptera: Formicidae), *Formica selysi* workers (subfamily Formicinae), *Manica rubida* workers, and *Myrmica rubra* queens, workers, and larvae (both Myrmicinae), one species of beetle (Coleoptera: Tenebrionidae), *Tenebrio molitor* adults, and one species of fly (Diptera: Calliphoridae) *Calliphora vomitoria* adults were used in these experiments.

Pieces of cuticle from the head, thorax, or abdomen were dissected under a binocular microscope, freed from any internal tissue using fine forceps, then sealed in soft glass capillary tubes (Morgan, 1990). Legs, wings, and antennae were pulled off with fine forceps and directly sealed in soft glass capillaries (15 × 20 mm × 1.8 mm and 0.2 mm wall thickness). Larvae of *M. rubra* were

slit open, the gut removed, and the body cavity rinsed with water, squeezed to remove the water, and sealed in a capillary in the same way.

Gas chromatography–mass spectrometry was carried out with a Hewlett-Packard 5890 gas chromatograph directly coupled to a 5970B Mass Selective Detector (quadrupole mass spectrometer using 70 eV electron impact ionization). The system was controlled by, and data accumulated on, a Hewlett-Packard Series 300 computer with HP 59970C Chemstation. Mass spectra were scanned from *m/z* 35 to *m/z* 550. Scan time was about 2.4 sec.

Chromatography was performed on an immobolized polydimethylsiloxane phase (equivalent to OV-1) in a fused silica column (12 m × 0.2 mm, 0.33 μm film thickness, SAC Chromatography, Letchworth) linked to the mass spectrometer source through a 10-m length of 0.2-mm-ID, deactivated fused silica capillary (SAC Chromatography). Helium was used as carrier gas at 1 ml/min. The sample was heated in the injector to 250°C for 4 min before crushing the capillary as described originally by Morgan and Wadhams (1972). An illustration and description of the injector in its current form is given by Morgan (1990). The oven was programmed from 150°C at 3°C/min to 275°C and then held isothermal for varying lengths of time. The split vent was closed before crushing the sample and reopened 30 sec later.

A standard alkane–alkene mixture from C_{18} to C_{30} was prepared in hexane (100 ng/μl) as a retention time and quantitative standard. Identification of substances is based upon mass spectrometry and comparison with published results.

Preliminary experiments were made first by syringe injection, in the split mode, of a solution of hydrocarbons to discover the best temperature program for separation of the high-molecular-mass alkanes and alkenes. Then 1-μl quantities of the standard hydrocarbon solution were placed in the soft glass capillaries and, after allowing most of the hexane to evaporate, they were sealed, heated, and crushed in the gas chromatograph, using the solid sampler (Morgan and Wadhams, 1972). The temperature of the injector and the time of heating were varied until good peak shapes were obtained for all, including the last-eluted peaks, and their peak-area ratios corresponded to those from the syringe injection. It was found that a temperature of 250°C and 4 min of heating was more than sufficient to completely elute triacontane so these conditions were adopted for further work.

RESULTS

Separate chromatograms were obtained from the abdominal cuticle, thorax, antenna, and legs of a single worker of *Formica selysi*. The chromatogram from a single leg is shown in Figure 1. That of the thorax was practically identical. A chromatogram of a fragment of abdominal cuticle is illustrated in Bagnères

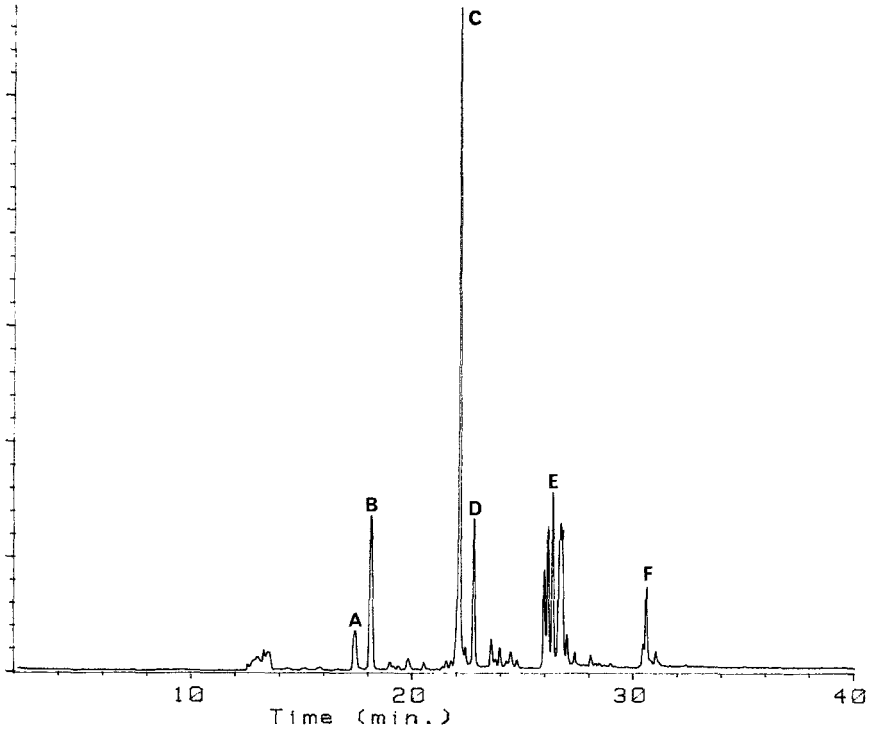


FIG. 1. Chromatogram of the cuticular hydrocarbons from two legs of a worker of the ant *Formica selysi* by the solid injection technique. The labeled peaks are: A, 9-pentacosene; B, pentacosane; C, 9-heptacosene; D, heptacosane; E, 6,20-nonacosadiene; F, mixture of 9,21- and 8,22-hentriacontadiene. This sample was analyzed with a different temperature program from the other figures.

and Morgan (1990). These chromatograms should be compared with those obtained by solvent extraction of whole bodies obtained from the same species (Bagnères et al., 1990). In each, the major substances are pentacosane, 9-heptacosene, 6,20-nonacosadiene, and 9-nonacosene (Figure 1), but all the minor components found by Bagnères et al. (1990) are present in approximately the same proportions.

From *Manica rubida* almost identical simple patterns were obtained from legs and abdomen. A chromatogram from a single leg is shown in Figure 2. The mixture is dominated by tricosane, pentacosane, and heptacosane. A slightly more complex pattern with more of the minor components was obtained from the antennae. This chromatogram is reproduced in Bagnères and Morgan (1990). These again may be compared with the solvent-extracted profiles for *M. rubida* published by Bagnères et al. (1990).

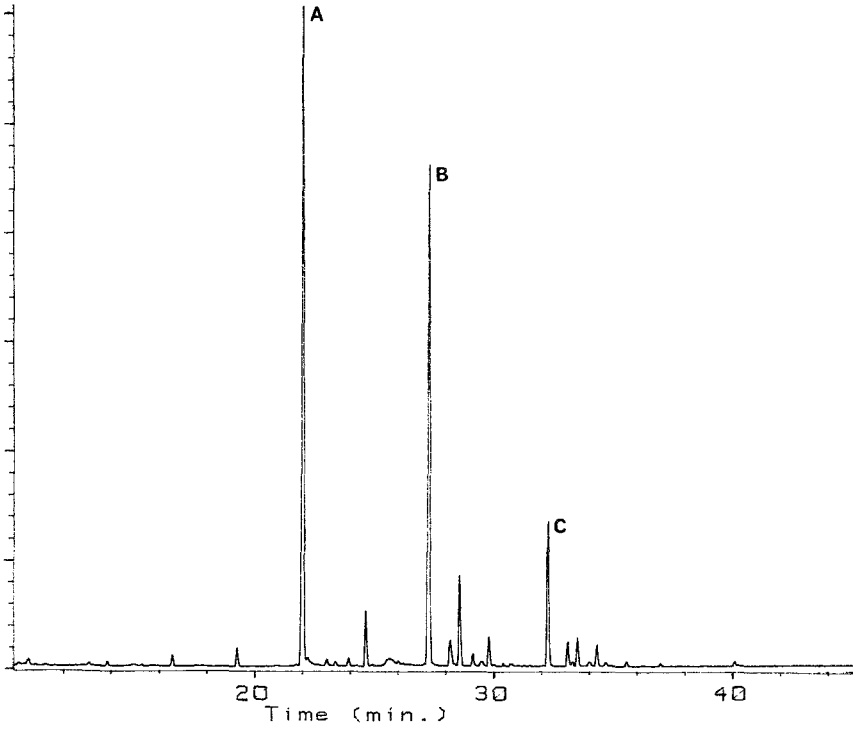


FIG. 2. Chromatogram of the cuticular hydrocarbons from a single leg of a worker of the ant *Manica rubida* by the solid injection technique. The labelled peaks are: A, tricosane; B, pentacosane; C, heptacosane.

Myrmica rubra was examined in greater detail. We already have shown that the cuticle of queen and worker are very similar but quantitatively different from that of larvae (Bagnères and Morgan, 1990). The cuticle of *M. rubra* larvae contains a much higher proportion of pentacosane. We have compared the chromatograms obtained from legs, abdomen, and antennae of *M. rubra* workers here and find the same substances present in each portion of the body, but the proportion of pentacosane to the other components varies from one individual to another. In several examples, the most complex pattern was obtained from the abdomen or gaster. One of these is illustrated in Figure 3. The substances identified are listed in Table 1, together with the diagnostic mass spectral fragment ions used for the identification of the methyl-branched hydrocarbons and the equivalent chain length for each substance. The pattern in Figure 3 can be compared with the solvent-extract gas chromatogram of *M. rubra* reproduced by Vienne et al. (1990).

In Figure 4 are reproduced chromatograms of the wings, abdomen, and

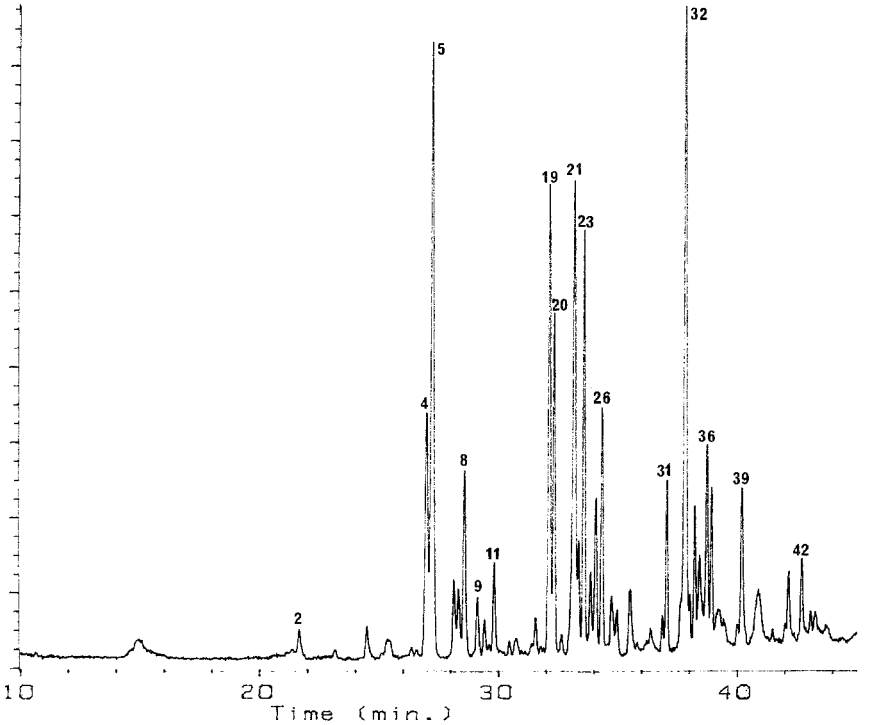


FIG. 3. Chromatogram of the cuticular hydrocarbons from a part of the abdomen (gaster) of a single worker of the ant *Myrmica rubra*, obtained by the solid injection technique. The identification of the numbered peaks is given in Table 1.

TABLE 1. COMPOUNDS IDENTIFIED BY GC-MS ON CUTICLE OF *Myrmica rubra* WORKERS

No.	Compound[a]	Diagnostic ions	Mol. mass	Equivalent chain length
1	11-Me + 8-Me + 7-MeC_{21}	168/169; 140/141, 196/197; 112/113, 224/225	310	21.40
2	Tricosane		324	23.00
3	11-Me + 9-Me + 7-MeC_{23}	168/169, 196/197; 140/141, 224/225; 112/113, 252/253	338	23.40
4	Pentacosene		350	24.90
5	Pentacosane		352	25.00
6	13-Me + 11−MeC_{25} (+9−MeC_{25})	196/197; 168/169, 224/225	366	25.37
7	7−MeC_{25}	112/113, 280/281	366	25.44
8	5−MeC_{25}	84, 308/309	366	25.50
9	9,13−DiMeC_{25}	140, 196, 211, 267	380	25.66
10	3−MeC_{25}	56, 336/337	366	25.71

TABLE 1. Continued

No.	Compound[a]	Diagnostic ions	Mol. mass	Equivalent chain length
11	5,11−$DiMeC_{25}$	84, 183, 224, 324	380	25.83
12	Hexacosane		366	26.00
13	x,y−$DiMeC_{26}$		394	26.14
14	14−Me + 12−Me + 10−MeC_{26}	210/211, 196/197; 184/185, 224/225, 154/155, 252/253	380	26.38
15	6−MeC_{26}	98/99, 308/309	380	26.45
16	5−MeC_{26}	84, 322/323	380	26.49
17	10,12−$DiMeC_{26}$	154, 267, 196, 225	394	26.65
18	6,10−$DiMeC_{26}$	98, 113, 169, 323	394	26.76
19	Heptacosene		378	26.89
20	Heptacosane		380	27.00
21	13−Me + 11−MeC_{27}	196/197, 224/225; 168/169, 252/253	394	27.36
22	7−MeC_{27}	112/113, 308/309	394	27.45
23	5−MeC_{27}	84, 336/337	394	27.60
24	11,15−$DiMeC_{27}$	168, 196, 268, 238	408	27.69
25	3−MeC_{27}	56, 364/365	394	27.76
25′[b]	7,16−$DiMeC_{27}$	112, 182, 252, 323	408	27.76
26	5,11−$DiMeC_{27}$	84, 183, 252, 352	408	27.89
27	Octacosane		394	28.00
27′	x,y−$DiMeC_{28}$		422	28.01
28	14−Me + 13−Me + 12−Me + 10−MeC_{28}	224/225, 210/211; 196/197, 224/225; 182/183, 252/253; 154/155, 280/281	408	28.31
29	(10,12)−$DiMeC_{28}$		422	28.55
30	Nonacosene		406	28.83
31	Nonacosane		408	29.00
32	13−Me + 11−MeC_{29}	196/197, 252/253; 168/169, 280/281	422	29.34
33	7−MeC_{29}	112/113, 336/337	422	29.41
34	5−MeC_{29}	84, 364/365	422	29.50
35	13,17−$DiMeC_{29}$(+x)	196/197, 266/267	436	29.62
36	3−MeC_{29}	56, 392/393	422	29.75
36′	x,y−DiMe		436	29.75
37	5,11−$DiMeC_{29}$	84, 182, 281, 379	436	29.82
38	Triacontane		422	30.00
39	15−Me + 13−Me + 11−MeC_{29}	224/225, 238/239; 196/197, 266/267; 168/169, 294/295	436	30.30
40	5−MeC_{30} (+ silicone)	84, 378/379	436	30.50
41	15−Me + 13−Me	224/225, 252/253; 196/197, 280/281; 168/169, 308/309	450	31.30
42	13,17−$DiMeC_{31}$	196, 295, 224, 267	464	31.60

[a] x is unknown component. x,y−DiMe: Position of double bonds in alkenes has not been determined.
[b] Peak 25′ is a shoulder on peak 25, etc.

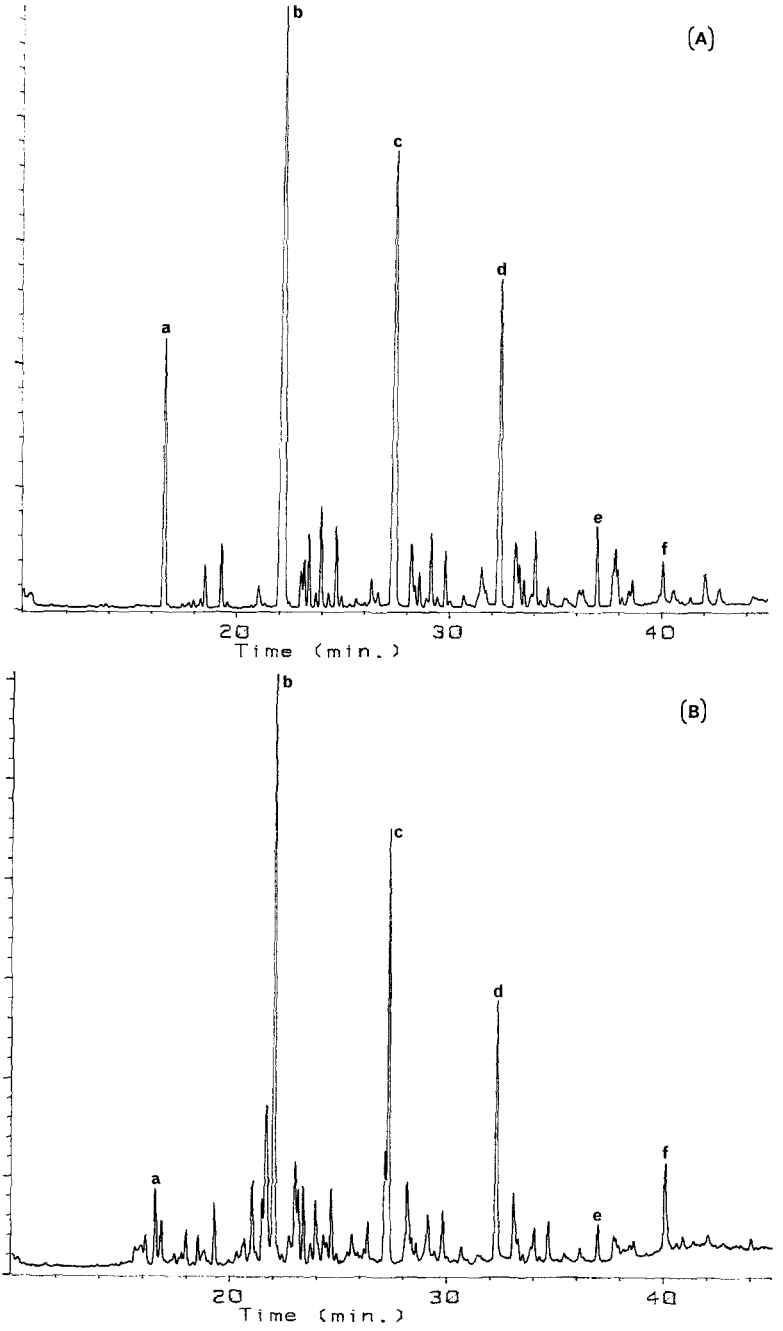


FIG. 4. Chromatograms obtained by the solid injection technique of the cuticular lipids on two wings (A), a piece of the abdomen (B), and one leg (C) of a single adult of the dipteran *Calliphora vomitoria*. The labelled peaks are a, heneicosane; b, tricosane; c, pentacosane; d, heptacosane; e, nonacosane; f, cholesterol.

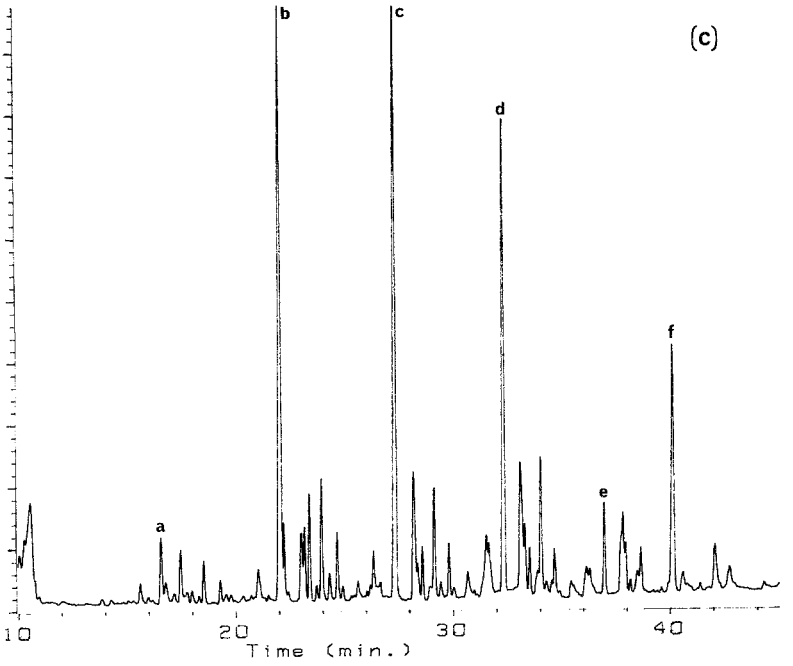


FIG. 4. Continued

legs of an adult *Calliphora vomitoria*. The predominance of tricosane, pentacosane, and heptacosane in each is apparent, as well as the variable minor components. The presence of significant quantities of cholesterol on the abdomen and legs is noteworthy. Mass spectra were obtained for each peak but the detailed identifications except for cholesterol have already been published (Trabalon et al., 1988a).

Chromatograms of selected body parts of an adult *Tenebrio molitor* are shown in Figure 5. The qualitatively similar and quantitatively varying pattern of hydrocarbons on elytra (wing covers), antenna, and an abdominal segment are seen. As well as the major hydrocarbons, small amounts of oxygenated compounds (fatty esters, alcohols, and ketones) were also present on the cuticle of this insect, but had not been found on the other species examined. The solvent-extracted hydrocarbons from the elytra and wings of adult *T. molitor* were reported by Lockey (1978). While we identified many of the substances, we found different proportions in the substances with much more 11-methyltricosane. Lockey (1978) did not find the oxygenated compounds because of the chromatographic steps used to purify the hydrocarbons.

To give some indication of quantity, the two legs of *F. selysi* shown in Figure 1 were covered with 1.2 μg of hydrocarbons, the one leg of *M. rubida*

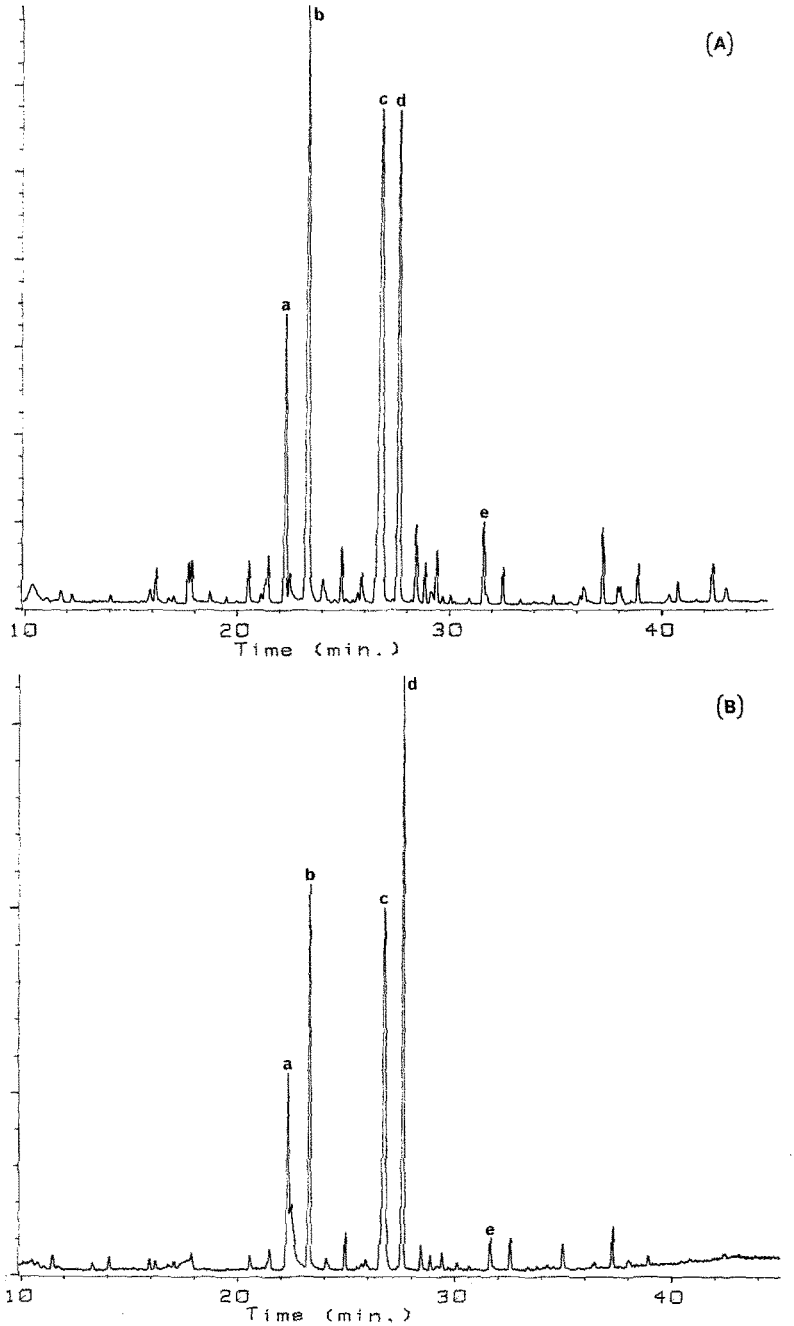


FIG. 5. Chromatograms obtained by the solid injection technique of the cuticular lipids on a piece of the elytra (A), one antenna (B), and a single leg (C) of an adult *Tenebrio molitor*. Labeled substances are: a, tricosane; b, 11-methyltricosane; c, mixture of 9-pentacosene and 10-pentacosene; d, pentacosane; e, heptacosane.

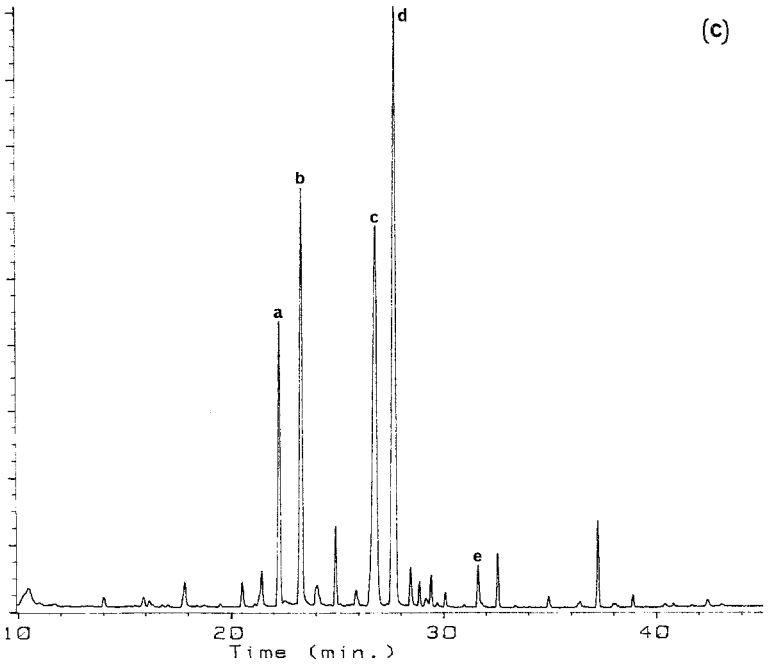


FIG. 5. Continued

in Figure 2 was coated with 0.84 μg. *C. vomitoria* (Figure 4C) and *T. molitor* (Figure 5C) were found to have 4 μg and 5 μg per leg, respectively, as would be expected from these much larger insects. The two wings of *C. vomitoria* (Figure 4A) carried 9.4 μg of hydrocarbons. The single antenna of *T. molitor* (Figure 5B) was covered with 0.6 μg. The gaster of *M. rubra* in Figure 3 yielded 1.0 μg; other samples gave up to 3.2 μg hydrocarbons per gaster. No attempt has been made now to obtain mean values from a large number of samples. Quantities 10 to 100 times less could easily be quantified.

DISCUSSION

Because of the great current interest in insect cuticular hydrocarbons, and because we believe this method is simpler, more sensitive, and less liable to contamination from other secretions, we have demonstrated here the possibility of direct vaporization of hydrocarbons from the cuticle. Because our objective has been chiefly to demonstate the practicability of the method, only a small part of the studies we have made are described.

From the various samples examined, it can be shown, first, that the removal is quantitative, since the pattern of substances is the same as obtained by solvent extraction, and there is no bias towards more volatile substances. Second, the

method can be applied to single insects, so that a species-characteristic pattern can be obtained from a single specimen. Third, for the five species studied, qualitatively, the same hydrocarbon mixtures are found on all parts of the body. Fourth, these principles apply both to social and nonsocial insects from three Orders. Fifth, the method is sufficiently sensitive, and the amounts of these hydrocarbons sufficiently large, that a single antenna or a mandible provides material for a gas chromatogram and mass spectra of the components.

Our primary interest is in ants, but to illustrate the universality of the method we have examined one dipteran (*Calliphora vomitoria*) and one coleopteran (*Tenebrio molitor*). It is interesting to see that the wings of *C. vomitoria* contain the same cuticular wax as other parts of the body. In the example of *T. molitor*, we have shown that the antenna and fragments of the elytra of this species are equally amenable to direct solid-sampling gas chromatography.

It is well known that there are age, seasonal, and sexual differences in the hydrocarbon pattern on insect cuticle (cf. Jackson and Blomquist, 1976; Blomquist et al., 1987; Trabalon et al., 1988b). In the one species we examined in greater detail, *M. rubra*, we found differences between larvae and workers and less pronounced differences between queens and workers (Bagnères and Morgan, 1990), but we find essentially the same mixture on all body parts of a particular caste. Larvae of *M. rubra* have a relatively hard waxy coating, because of the large amount (about 670 ng per larva, 38% of the total) of the solid hydrocarbon, pentacosane (mp 54°C) on their cuticle.

Insects have an important and effective method of antennal perception, requiring only 0.1 sec of contact (Clément, 1982). The work of Bonavita-Cougourdan et al. on *Camponotus vagus* (1987) (Formicidae) and of Bagnères et al. (in preparation) on *Reticulitermes* (Isoptera) have shown that the cuticular hydrocarbons are used as signals of recognition. The cuticular substances function as contact pheromones, and the study of their chemical composition is a necessary part of understanding the process of chemical communication. Until now, it has not been practical to study the cuticle of single individuals in their social interactions. We believe the use of this technique will help to advance our knowledge of this subject.

We have not exhausted the scale of the method; still smaller segments of cuticle, individual sclerites, or even antennal or leg segments can be examined by GC-MS by the solid-sampling technique.

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GUT REDOX CONDITIONS IN HERBIVOROUS LEPIDOPTERAN LARVAE

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Abstract—Large interspecific differences in redox potential exist among herbivorous lepidopteran larvae. Reducing conditions occur in the midguts of *Manduca sexta* (Sphingidae) and *Polia latex* (Noctuidae), whereas oxidizing conditions prevail in the midguts of *Lymantria dispar* (Lymantriidae), *Danaus plexippus* (Danaidae), and *Papilio glaucus* (Papilionidae). The epithelium of the posterior midgut of *M. sexta* fed a diet containing bismuth subnitrate accumulates bismuth sulfide, suggesting that sulfide might be one of the reducing agents responsible for the maintenance of reducing conditions in this species. We propose that the effects of plant allelochemicals in insect herbivores will be strongly affected by gut redox conditions and that the regulation of gut redox conditions is an important adaptation of insect herbivores to the chemical defenses of plants. The redox state of the gut is yet another insect trait that must be included in the analysis of plant–insect interactions.

Key Words—Plant–insect interactions, redox, reduction potential, digestion, Lepidoptera, *Danaus plexippus*, *Lymantria dispar*, *Manduca sexta*, *Papilio glaucus*, *Polia latex*, Danaidae, Lymantriidae, Sphingidae, Papilionidae, Noctuidae.

INTRODUCTION

The redox conditions that prevail in an insect's gut can have a major impact on the digestion, metabolism, and assimilation of ingested nutrients. Highly reducing conditions (negative redox potentials) in the midguts of tineid moths (Water-

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house, 1952b), dermestid beetles (Waterhouse, 1952c), and bird lice (Waterhouse, 1953) facilitate the digestion of keratin, the major protein of fur and feathers, which is highly resistant to digestion by most animals. Under reducing conditions the disulfide bonds that stabilize keratin are cleved, resulting in the liberation of polypeptide subunits that are readily degraded by insect digestive proteases. Reducing conditions are also commonly encountered in portions of the gut that house large populations of metabolically active microorganisms. Negative redox potentials have been reported in the hindguts of termites (Veivers et al., 1980; Bignell, 1984), roaches (Day and Waterhouse, 1953; Bignell, 1984), and scarab beetles (Bayon, 1980), where symbiont-mediated fermentative digestion of cellulose occurs.

Gut redox conditions are also likely to mediate the impact of many classes of ingested allelochemicals on herbivorous insects. For example, the redox state of the alimentary tract is likely to determine whether ingested phenols remain in the reduced state or are oxidized to quinones, which are generally more reactive and more toxic substances than phenols. Thus, gut redox conditions are a potential adaptive mechanism by which an insect herbivore might minimize the adverse effects of ingesting the defensive chemicals present in its food plant. Prior to this study, there had been only one measurement of gut redox potential in an herbivorous insect. Bignell (1984) reported oxidizing conditions throughout the gut of *Locusta migratoria*. In the present study we have assessed the redox conditions in the alimentary tracts of larvae of five species of herbivorous Lepidoptera: the tobacco hornworm, *Manduca sexta* (Sphingidae); the cutworm *Polia latex* (Noctuidae); the gypsy moth, *Lymantria dispar* (Lymantriidae); the common monarch butterfly, *Danaus plexippus* (Danaidae); and the black swallowtail, *Papilio glaucus* (Papilionidae). In reporting and discussing our results, we have borrowed heavily from approaches developed by investigators studying the redox properties of soils.

METHODS AND MATERIALS

Oxidation–Reduction Potentials. A redox reaction is one in which there is a transfer of electrons, with or without the accompanying transfer of protons (reaction 1):

$$\text{oxidized form} + n\text{H}^+ + ne^- = \text{reduced form} \qquad (1)$$

Consequently, whether a redox couple exists primarily in its oxidized or reduced form depends upon the availability of both protons and electrons in the system. Proton availability in a system is a function of its acid–base status. Electron availability is a function of the tendencies of the various chemical species pres-

ent to lose electrons (and act as reducing agents) or gain electrons (and act as oxidizing agents). In a system in which proton activity and electron availability are high, reduction is favored; if proton activity and electron availability are low, oxidation is favored. If electron availability is held constant, oxidation is more favored under alkaline conditions than under acidic conditions.

The assessment of the redox conditions in an insect's gut requires a measurement of both acid–base status and electron availability. The acid–base status of the gut can, of course, be determined by a simple pH measurement. However, the measurement of electron availability in a complex aqueous mixture is more problematic. The standard approach has been to determine the redox potential by measuring the emf of a platinum electrode inserted into the gut and to add to the observed potential the potential difference between the reference electrode and the standard hydrogen electrode. Although redox potentials measured in this way routinely are called "standard reduction potentials" and designated by the symbol "E_h," which implies that they are equilibrium redox potentials, they are actually mixed potentials that are the resultants of all of the redox couples present in the system, most of which are not at equilibrium. The contribution of each redox couple present in the mixture is an unknown function of its concentration and its reversibility. Such mixed potentials are, therefore, only rough measures of electron availability.

In describing the redox conditions in insect guts, we have used terms developed by soil scientists to characterize the redox conditions of soils (Lindsay, 1979; Bohn et al., 1985; Bartlett, 1986). Electron availability, or "pe," is derived from the Nernst equation, and at 25°C, is defined as:

$$\text{pe} = E_h/59.2$$

where E_h is the observed standard reduction potential in millivolts. Just as pH expresses the negative log of proton activity, pe expresses the negative log of electron activity. On either the pH or pe scales, zero is the standard state, and a unit of pH or pe is equivalent to 2.3 RT joules of free energy per mole of protons or electrons, respectively. A low pe system has a surplus of electrons and, therefore, a high tendency to lose electrons and become more oxidized. A high pe system has a deficiency of electrons and a high tendency to gain electrons and become more reduced.

To characterize the redox status of a complex aqueous system, Lindsay (1979) has suggested using the sum of pH and pe as a convenient single term expression that accords equal weight to acid–base status and electron availability. He has defined pe + pH as the "redox parameter." By taking the logarithm of the expression for the equilibrium constant of a generalized redox half-reaction, such as reaction 1, in which the activities of the oxidized and reduced

species are equal, and substituting pH and pe for their defined equivalents, it follows that

$$\text{pe} + \text{pH} = (1/n) \log K$$

Thus, for any given system at equilibrium, which is probably not actually the case for either soil samples or insect gut contents, pe and pH should change in a compensatory manner such that pe + pH remains constant. In an aqueous system, in which the maximum oxidizing and reducing capacities are set by the water–oxygen and hydrogen–proton couples, respectively, pe + pH can range from a high value of 20.78 (highly oxidizing conditions) to a low value of 0 (highly reducing conditions).

Insects. Fifth-instar *Manduca sexta* were reared from eggs (Carolina Biological Supply) on an artificial diet (Bio-Serv #9783) in a growth chamber at 25°C with a 16:8 light–dark photoperiod. Sixth-instar *Lymantria dispar* were reared from sterilized egg masses of the New Jersey-OTIS strain supplied by the USDA-ARS (APHIS, Otis AFB, Massachusetts) on artificial diet prepared according to ODell and Rollinson (1966), but lacking casein, in a growth chamber at 25°C with a 16:8 light–dark photoperiod. Fifth-instar *Papilio glaucus* were obtained from a lab culture (J.M. Scriber, Michigan State University) reared on excised foliage of tulip tree (*Liriodendron tulipifera*). Final instars of *Polia latex* and *Danaus plexippus* were collected from black walnut (*Juglans nigra*) and common milkweed (*Asclepias syrica*) foliage, respectively, in Centre County, Pennsylvania, and were fed excised foliage of their host until measurements of gut pH and E_h were conducted.

Gut pH, E_h, and pe. Three to five larvae of each species were immobilized on ice or by exposure to diethyl ether, and the gut was exposed by a ventral longitudinal incision. The integument was folded back and secured away from the gut using insect pins, and hemolymph was blotted from the integument and gut surfaces. The preparation of a larval gut required less than a minute. If dissection resulted in puncture of the gut wall, or if the gut was not full, larvae were discarded. In *M. sexta*, the shortness of the foregut precluded the accurate measurement of foregut pH and E_h in all but one larva.

A longitudinal profile of gut redox potential was made using a 0.02 in. platinum electrode (Microlectrodes MI-800) and a silver–silver chloride microreference electrode (Microelectrodes MI-401) connected to a Metrohm/Brinkmann (model 103) or an Accumet (model 291) millivolt and pH meter. Potential measurements were made in the foregut, anterior midgut, medial midgut, posterior midgut, and hindgut. Redox potentials generally stabilized at each location within 10 sec, such that measurements on an individual larva were completed within a minute. The observed redox potentials (E_{obs}) were converted to standard redox potentials (E_h) by adding 200 mV, which is the poten-

tial difference between the silver–silver chloride and hydrogen reference electrodes. Calculation of pe was from E_h, using the equation pe = $E_h/59.2$ (Lindsay, 1979). Following the measurement of redox potential, a longitudinal profile of gut pH was made using a micro-needle pH electrode (Microelectrodes MI-408c) and a silver–silver chloride reference electrode (Microelectrodes MI-401). pH values generally stabilized at each location of the gut within a minute, such that all measurements on an individual larva were completed within 5 min. In *P. glaucus* and *D. plexippus*, there was some regurgitation of midgut fluid into the foregut in some animals, which compromised the accuracy of the measurements of foregut pH and E_h.

Mean values of pH, E_h, pe, and pe + pH were compared by ANOVA. Significant pairwise differences were determined by LSD analysis. When variances were not equal, treatment means were compared by the Kruskal-Wallis test, and the significance of pairwise differences was determined by the median test with alpha set at 0.01 to adjust for multiple comparisons.

Test for Sulfide in Midgut. M. sexta and *L. dispar* larvae, reared from eggs on artificial diets, were switched early in the fourth instar to a portion of the same artificial diet that had been amended by the addition of bismuth subnitrate (10% dry weight). After 24 hr, three larvae of each species were immobilized and the gut exposed as described above. The presence of sulfide ions in the gut contents or gut epithelium was indicated by a black precipitate of bismuth sulfide.

RESULTS AND DISCUSSION

Previous Reports of Redox Conditions in Insect Guts. We have calculated pe and pe + pH at various gut locations for all insect species for which we could find measurements of pH and E_h in the literature (Table 1). In most studies of gut redox conditions in insects, the results are reported as a range, not as a mean. In Table 1, we have reported the range of reported E_h values and have calculated values for pe and pe + pH based on midrange values. Midrange values for pe and pe + pH in insects range from −4.4–5.1 and 2.8–11.6, respectively. To provide a measure of the most extreme reducing conditions encountered in insect guts, we also have calculated the lowest values for pe and pe + pH observed in those species that possess gut regions with high electron availabilities.

High electron availability (pe < -3.0) is characteristic of the hindguts of termites and the midguts of the bird lice *C. columbae* and *E. stramineus*, the dermestid beetles, and the webbing clothes moth *T. bisselliella*. Of the species listed in Table 1, the conditions most conducive to reduction (pe + pH < 4.5)

TABLE 1. COMPILATION OF pH, E_h, pe, AND pe + pH FROM EARLIER STUDIES OF REDOX CONDITIONS IN INSECT GUTS[a]

Order and species	Gut location	pH	E_h (mV)	pe		pe + pH	
				Midrange	Lowest	Midrange	Lowest
Thysanura							
Ctenolepisma lineata[b]	MG	6.4–7.0	+290 to +170	3.9		10.5	
	HG	2.6–3.8	+160	2.7		5.9	
Orthoptera							
Blattella germanica[b]	MG	6.0–6.2	+30 to +10	0.3		6.4	
	HG	8.0	−90 to −120	−1.7	−2.0	6.3	6.0
Periplaneta americana[c]	FG	5.4–6.5	+347 to +252	5.1		11.0	
	MG	6.1–6.7	+157 to +37	1.6		8.0	
	HG	6.3–6.7	+167 to −173	−0.1	−2.9	6.4	3.6
Locusta migratoria[d]	FG	5.7–6.6	+327 to +77	3.4		9.5	
	MG	6.4–7.4	+267 to +67	2.8		9.7	
	HG	5.8–6.8	+287 to +97	3.2		9.5	
Isoptera							
Nine termite species[e]	MG	7.0	> +100	>1.7		>8.7	
	HG	7.0	−230 to −270	−4.2	−4.5	2.8	2.5
Cubitermes severus[f]	FG	6.7	+347 to +187	4.5		11.2	
	HG(P_1)	10.4	+27 to −183	−1.3	−3.1	9.1	7.3
	HG(P_3)	9.2	−13 to −123	−1.1	−2.1	8.1	7.1
	HG(P_{4a})	8.2	+87 to −98	−0.1	−1.7	8.1	6.5
	HG(P_{4b})	8.0	+197 to −53	1.2	−0.9	9.2	7.1
Zootermopsis nevadensis[f]	FG	6.8	+257 to +157	3.5		10.3	
	MG	7.1	+207 to +137	2.9		10.0	
	Paunch	7.1	−73 to −303	−3.2	−5.1	3.9	2.0
	Colon	7.3	+147 to −163	0.1	−2.8	7.2	4.5

Mallophaga							
Columbicola columbae[g]	aMG	7.8–8.0	−170 to −215	−3.2	−3.6	4.7	4.3
	pMG	6.5	About −100	−1.7		4.8	
	HG	5.8	> +70	>1.2		>7.0	
Eomenacanthus stramineus[g]	aMG	8.0–8.4	−175 to −220	−3.3	−3.7	4.9	4.5
	pMG	6.4–7.2	About −115	−1.9		4.9	
	HG	6.4–7.2	> +30	>0.5		>7.3	
Damalina ovis[g]	MG	6.5	> +77	1.3		8.8	
	HG	5.0–6.0	> +97	1.6		7.1	
Coleoptera							
Three dermestid species[h]	MG	6.8–8.2	−190 to −230	−3.5	−3.9	4.0	3.6
	HG	4.4–4.8	+260	4.4		9.0	
Oryctes nasicornis[i]	MG	11.8	+27 ± 18	0.5		11.6	
	HG	7.8–8.5	−83 ± 22	−1.4	−1.6	6.6	6.4
Lepidoptera							
Tineola bisselliella[j]	aMG	8.9–9.0	About −200	3.4		5.6	
	mMG	9.8–10.0	−220 to −300	−4.4	−5.1	5.5	4.8
	pMG	6.2–6.5	+80 to +30	1.8		8.2	
	HG	4.5–5.8	> +250	4.2		9.6	
Hymenoptera							
Apis mellifera[k]	MG	7.5	+257 to −83	1.5		9.0	

[a] Abbreviations: FG, foregut; MG, midgut; aMG, anterior midgut; mMG, medial midgut; pMG, posterior midgut; HG, hindgut.
[b] Day and Waterhouse (1953).
[c] Redox data from Bignell (1984); pH data from Bignell (1981).
[d] Redox data from Bignell (1984); pH data from Srivastava and Srivastava (1956) and Bodine (1925).
[e] Veivers et al. (1980); pH values assumed, not measured; termite species were *Ceratokalotermes spoilator, Coptotermes lacteus, Glyptotermes brevicornis, Incistitermes barretti, Mastotermes darwiniensis, Nasutitermes exitosus, Neotermes insularis, Porotermes adamsoni,* and *Stolotermes victoriensis.*
[f] Bignell (1984); P_1, P_3, P_{4a}, P_{4b}, paunch and colon are distinct segments of the hindgut.
[g] Waterhouse (1953).
[h] Waterhouse (1952c); species were *Anthrenocerus australis, Anthrenus verbasci,* and *Attagenus piceus.*
[i] Bayon (1980).
[j] Waterhouse (1952b).
[k] Bignell and Heath (1985).

occur in the hindguts of termites and the midguts of dermestid beetles. For comparison, a flooded soil containing decomposable organic matter, with a value of pe + pH below 4.5, would be classified as a highly anaerobic soil and might be expected to accumulate methane (Rowell, 1988). Methanogenesis is a process that is known to occur in the hindguts of many termite species (Breznak, 1984). Somewhat less extreme reducing conditions (pe + pH = 4.5–7.0) occur in the hindguts of the silverfish *C. lineata*, the two roach species, the soil-feeding termite *C. severus*, the rhinocerus beetle *O. nasicornis*, and in the midguts of the German cockroach *B. germanica*, the two species of bird lice, and the webbing clothes moth. For comparison, a waterlogged organic soil with a value of pe + pH between 4.5 and 7.0 would be classified as a reducing soil and might be expected to accumulate sulfide (Rowell, 1988). Sulfide appears to be one of the reducing agents responsible for the maintenance of high electron availability in the midgut of the webbing clothes moth (Waterhouse, 1952a; Yoshimura et al., 1988).

Redox Conditions in Gut Fluids of Herbivorous Caterpillars. Reducing conditions exist in the midguts of *M. sexta* and *P. latex* (Table 2). As in *T. bisselliella*, the reducing conditions in the midguts of *M. sexta* and *P. latex* reflect the presence of high electron availabilities (negative values of pe), which compensate for low proton availabilities (high values of pH). In all three of these lepidopteran species, the lowest proton availability (highest pH), the highest electron availability (most negative pe), and the most reducing conditions (lowest values of pe + pH) occur in the anterior and medial portions of the midgut (Table 2). Although the conditions in the midguts of *M. sexta* and *P. latex* are reducing, they are somewhat less so than those in the midguts of the webbing clothes moth and the three dermestid beetles and than those in the hindguts of nine termite species (Table 1). However, the midguts of these two herbivorous caterpillars are as reducing as the midguts of the bird lice *C. columbae* and *E. stramineus*, the hindguts of the cockroach *B. germanica*, the scarab beetle *O. nasicornis*, and the hindguts of two termite species. The redox conditions that exist in the midguts of *M. sexta* and *P. latex* are comparable to those of a waterlogged organic soil; that is, reducing but not highly anaerobic (Rowell, 1988).

Although the midguts of *M. sexta* and *P. latex* are strongly reducing, the redox conditions that exist elsewhere in the gut are weakly oxidizing or intermediate between oxidizing and reducing (Table 3). Values for E_h are uniformly positive in both the foreguts and hindguts, and values for pe and pe + pH fall in the ranges 2.1–2.9 and 8.6–8.7 in the foregut and 1.5–4.2 and 8.9–9.6 in the hindgut. For comparison, soil samples with values of pe and pe + pH comparable to those observed in the foreguts and hindguts of the larvae of these two lepidopteran species are transitional between anaerobic and aerobic conditions (Rowell, 1988).

TABLE 2. pH, E_h, pe AND pe + pH OF ANTERIOR, MEDIAL, AND POSTERIOR MIDGUTS OF FIVE SPECIES OF LEPIDOPTERAN LARVAE[a]

Species (N) and midgut location	pH	E_h	pe	pe + pH
Manduca sexta (5)				
Anterior	8.0 (0.46)[a]	−131 (66.6)[a,b]	−2.2 (1.13)[a,b]	5.8 (1.58)[a]
Medial	9.3 (1.33)[b]	−188 (26.1)[a]	−3.1 (0.44)[a]	6.1 (0.81)[a]
Posterior	8.2 (0.66)[a]	−88 (96.8)[b]	−1.5 (1.63)[b]	6.8 (1.38)[a]
LSD (0.05,12)	0.9	78	−1.3	1.5
Polia latex (4)				
Anterior	7.8 (0.68)[a]	−85 (62.1)[a,b]	−1.4 (1.05)[a,b]	6.4 (0.77)[a]
Medial	8.8 (0.15)[b]	−132 (51.1)[a]	−2.2 (0.86)[a]	6.5 (0.90)[a]
Posterior	8.3 (0.76)[a,b]	−55 (36.0)[b]	−0.9 (0.61)[b]	7.4 (0.70)[a]
LSD (0.05,9)	0.8	66	1.1	1.0
Lymantria dispar (5)				
Anterior	8.2 (0.38)[a]	+238 (45.1)[a]	4.0 (0.76)[a]	12.2 (0.70)[a]
Medial	8.2 (0.27)[a]	+214 (36.1)[a]	3.6 (0.61)[a]	11.8 (0.76)[a]
Posterior	7.9 (0.39)[a]	+218 (35.7)[a]	3.7 (0.61)[a]	11.6 (0.35)[a]
LSD (0.05,12)	0.4	44	0.7	1.1
Danaus plexippus (3)				
Anterior	8.6 (0.49)[a]	+77 (9.1)[a]	1.3 (0.15)[a]	9.9 (0.35)[a]
Medial	8.5 (0.15)[a]	+54 (4.9)[b]	0.9 (0.01)[b]	9.4 (0.11)[b]
Posterior	8.1 (0.31)[a]	+91 (8.6)[c]	1.5 (0.14)[c]	9.6 (0.40)[a,b]
LSD (0.05,6)	0.6	12	0.2	0.5
Papilio glaucus (3)				
Anterior	10.1 (0.50)[a]	+17 (58.6)[a]	0.3 (0.99)[a]	10.4 (0.98)[a]
Medial	10.1 (0.47)[a]	+48 (7.4)[a]	0.8 (0.12)[a]	11.0 (0.42)[a]
Posterior	9.8 (0.74)[a]	+57 (28.0)[a]	1.0 (0.47)[a]	10.8 (0.29)[a]
LSD (0.05,6)	0.9	179	1.0	1.0

[a] Values are means with standard deviations given in parentheses. In comparisons of different locations in the same species, values followed by the same letter are not significantly different at $P < 0.05$.

In contrast to *M. sexta* and *P. latex*, which have reducing midguts and mildly oxidizing foreguts and hindguts, *L. dispar*, *D. plexippus*, and *P. glaucus* larvae have guts in which oxidizing conditions prevail throughout (Tables 2 and 3). The average values of pe and pe + pH in the midguts of these species are in the ranges 0.7–3.8 and 9.5–11.9, respectively. Soils with these values for pe and pe + pH would be classified as aerobic soils (Rowell, 1988).

As the midgut is the primary site of digestion and assimilation in larval Lepidoptera, the large interspecific differences in midgut redox conditions reported here reflect significant differences in the chemical environment in which digestion and assimilation occur.

TABLE 3. pH, E_h, pe, AND pe + pH OF FOREGUTS, MIDGUTS, AND HINDGUTS OF FIVE SPECIES OF LEPIDOPTERAN LARVAE[a]

Species and gut location (N)	pH	E_h	pe	pe + pH
Manduca sexta				
Foregut (1)	5.7	+172	2.9	8.6
Midgut (5)	8.5 (0.61)^{a,1}	−136 (51.0)^{a,1}	−2.3 (0.86)^{a,1}	6.2 (0.99)^{a,1}
Hindgut (5)	7.4 (0.79)^{b,1}	+86 (48.0)^{b,1}	1.5 (0.81)^{b,1}	8.9 (0.85)^{b,1}
LSD (0.05,8)	0.8	58	1.0	1.1
Polia latex				
Foregut (4)	6.6 (1.27)^{a,1}	+123 (147.2)^{a,1}	2.1 (2.49)^{a,1}	8.7 (2.21)^{a,b,1}
Midgut (4)	8.3 (0.39)^{b,1}	−91 (43.6)^{b,1}	−1.6 (0.71)^{b,1}	6.7 (0.63)^{a,1}
Hindgut (4)	7.3 (0.92)^{a,b,1}	+99 (60.2)^{a,1,3}	1.7 (1.04)^{a,1,3}	8.9 (1.82)^{b,1,2}
LSD (0.05,9)	1.2	123	2.1	2.2
Lymantria dispar				
Foregut (5)	6.4 (0.41)^{a,1}	+236 (45.6)^{a,1}	4.0 (0.77)^{a,1}	10.5 (0.83)^{a,1}
Midgut (5)	8.1 (0.23)^{b,1}	+223 (36.4)^{a,2}	3.8 (0.61)^{a,2}	11.9 (0.56)^{b,2}
Hindgut (5)	7.1 (0.43)^{c,1}	+204 (36.6)^{a,2,3}	3.5 (0.62)^{a,2,3}	10.5 (0.51)^{a,2}
LSD (0.05,12)	0.4	45	0.8	0.8
Danaus plexippus				
Foregut (3)	7.5 (0.96)^{a,b,1,2}	+127 (73.5)^{a,1}	2.1 (1.25)^{a,1}	9.6 (0.42)^{a,1}
Midgut (3)	8.4 (0.06)^{a,1}	+74 (3.5)^{a,3}	1.1 (0.06)^{a,3}	9.5 (0.10)^{a,3}
Hindgut (3)	7.2 (0.52)^{b,1}	+114 (14.8)^{a,1}	1.8 (0.25)^{a,1}	9.0 (0.73)^{a,1,2}
LSD (0.05,6)	1.0	69	1.2	0.7
Papilio glaucus				
Foregut (3)	8.9 (1.42)^{a,b,2}	+122 (87.9)^{a,b,1}	2.1 (1.51)^{a,b,1}	11.0 (0.26)^{a,1}
Midgut (3)	10.0 (0.57)^{a,2}	+41 (26.0)^{a,3}	0.7 (0.40)^{a,3}	10.7 (0.40)^{a,2,3}
Hindgut (3)	7.6 (0.40)^{b,1}	+133 (18.9)^{b,1,3}	2.2 (0.32)^{b,1,3}	9.8 (0.10)^{b,1,2}
LSD (0.05,6)	1.4	86	1.4	0.5

[a] Values are means with standard deviations given in parentheses. Midgut values are the averages of the anterior, medial, and posterior midgut values reported in Table 2. In comparisons of different gut locations within a species, values followed by the same letter are not significantly different $P < 0.05$. In comparisons of the same gut locations between different species, values followed by the same number are not significantly different at $P < 0.01$.

Absorption of Bismuth Salts by Midgut Epithelium of M. sexta. When *M. sexta* larvae consumed artificial diet that contained bismuth subnitrate, the posterior midgut epithelium became dark brown (Figure 1), presumably because of the accumulation of bismuth sulfide in the cavities of the goblet cells. A similar accumulation of insoluble metal sulfides in the goblet cells of the midgut epithelium has been reported for the larvae of *T. bisselliella* fed a diet containing soluble metal salts (Waterhouse, 1952a). The accumulation of bismuth sulfide in the midgut epithelium of *M. sexta* and *T. bisselliella* larvae fed diets amended with bismuth subnitrate suggests that in both species sulfide may be

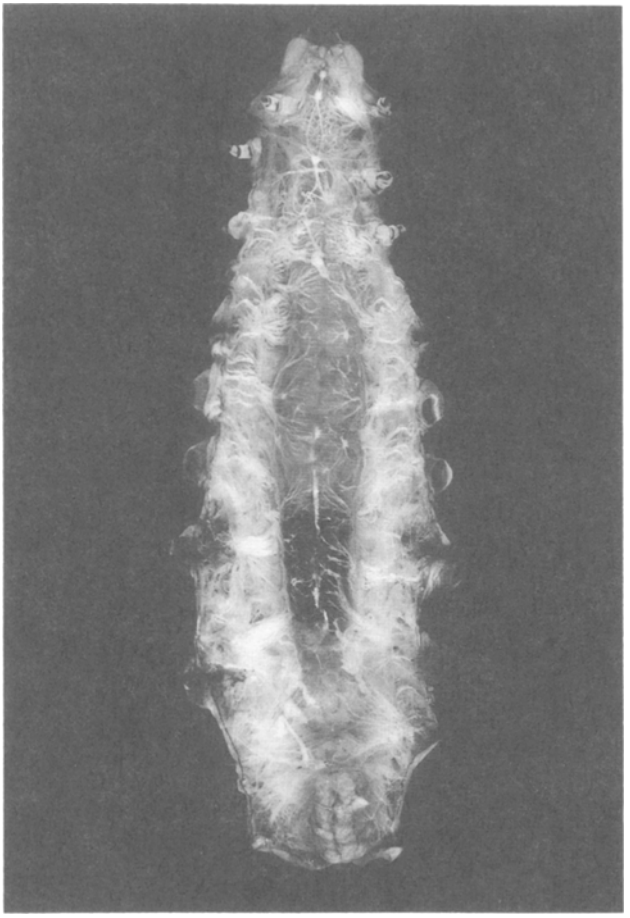


FIG. 1. Ventral view of the alimentary tract of a fourth-instar *M. sexta* larva that had been feeding on an artificial diet that included 10% bismuth subnitrate. The anterior of the larva is at the top of the photograph. The darkened region is the posterior third of the midgut. The dark substance, presumably bismuth sulfide, is in the midgut epithelium, not in the lumen contents.

one of the reducing agents responsible for the maintenance of reducing conditions. In *T. bisselliella* the sulfide is generated from cysteine by the action of L-cysteine lyase (Yoshimura et al., 1988).

No accumulation of bismuth sulfide was evident in the midgut epithelium of *L. dispar* larvae fed a diet amended with bismuth subnitrate.

Significance of Midgut Redox Conditions to Lepidopteran Herbivores. The redox state of the gut is likely to mediate the effects of many plant allelochem-

icals on insect herbivores. The impact of ingested phenols is likely to be especially dependent upon the redox conditions encountered in the gut, since quinones, which are oxidation products of phenols, are generally more reactive and more toxic than their phenolic precursors. Quinones can form covalent bonds with nucleophilic groups present in a variety of nutritionally important compounds (Leatham et al., 1980; Hurrell et al., 1982). The oxidation of foliar phenols, mediated by ingested foliar phenoloxidases, has been shown to reduce the efficiency of utilization of dietary amino acids and proteins in larvae of the tomato fruitworm, *Heliothis zea* (Lepidoptera, Noctuidae) (Felton et al., 1989). In addition, the toxic effects of plant phenols may depend upon the formation of highly reactive superoxide anion radicals during phenol oxidation. These reactive intermediates have been shown to inactivate enzymes, disrupt membranes, and damage DNA in mammals (Smith, 1985). Oxygen radicals, produced during the metabolism of phenols, may be responsible for the gut lesions observed in some insect herbivores maintained on diets rich in tannins (Bernays et al., 1980; Steinly and Berenbaum, 1985). Thus, both antinutritional and toxic effects of plant phenols may be strongly influenced by the redox conditions of the gut.

On the other hand, the oxidation of ingested phenols can have beneficial consequences for insect herbivores when the adverse effects on growth and fecundity are offset by the beneficial effects of reducing susceptibility to infection by microbial pathogens. The oxidation products of ingested phenols afford protection against the infection of *H. zea* by the nuclear polyhedrosis virus, HzSNPV (Felton and Duffey, 1990). Similarly, ingested phenols have been shown to protect the gypsy moth, *L. dispar*, against infection by the nuclear polyhedrosis virus GMNPV (Keating et al., 1989), although it has not yet been demonstrated that oxidized phenols are involved. Thus, it is possible that for one herbivore it would be advantageous to prevent phenol oxidation by the maintenance of reducing conditions in the midgut, whereas in another it would be advantageous to promote phenol oxidation by the maintenance of oxidizing conditions throughout the gut.

Tree-feeding caterpillars are more likely to encounter higher concentrations of tannins and other polyphenols than forb-feeding caterpillars. We were interested in determining whether there was a relationship between the growth form of the larval food plant and the redox conditions maintained in the midgut. We observed reducing conditions in one tree-feeder (*P. latex*) and one forb-feeder (*M. sexta*) and oxidizing conditions in two tree-feeders (*L. dispar* and *P. glaucus*) and one forb-feeder (*D. plexippus*). Thus, even this limited survey reveals that factors other than host plant growth form must account for the dichotomy between those Lepidoptera with oxidizing and those with reducing midguts.

We do not know yet whether ingested phenols undergo significant oxidation in the midguts of *L. dispar*, *D. plexippus*, and *P. glaucus*. Nor do we know

whether the conditions in the midguts of *M. sexta* and *P. latex* are sufficiently reducing to prevent the oxidation of ingested foliar phenols or to reduce ingested foliar quinones. Definitive resolution of those questions can be achieved only by determining the chemical fates of phenols of known structure that have been introduced into midgut fluids. On the basis of the findings reported in this paper, however, we feel justified in drawing the following conclusions: (1) the conditions in the midguts of *M. sexta* and *P. latex* are less conducive to the oxidation of ingested foliar phenols than the conditions in the midguts of *L. dispar*, *D. plexippus*, and *P. glaucus*; (2) the extent of oxidation of some foliar constituents will be greater in the midguts of *L. dispar*, *D. plexippus*, and *P. glaucus* than in the midguts of *M. sexta* and *P. latex*; and (3) some foliar constituents that are oxidized in the midguts of *L. dispar*, *D. plexippus*, and *P. glaucus* will not be oxidized in the midguts of *M. sexta* and *P. latex*. On the basis of these conclusions, we propose that the effects of plant allelochemicals on insect herbivores will be affected by gut redox conditions and that the regulation of gut redox conditions is an important adaptation of insect herbivores to the chemical defenses of plants. The redox state of the gut is yet another insect trait that must be included in the analysis of plant–insect interactions.

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OLFACTORY DISCRIMINATION ABILITY IN SHORT-TAILED FRUIT BAT, *Carollia perspicillata* (Chiroptera: Phyllostomatidae)

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Abstract—The olfactory discrimination ability in a fruit-eating bat, *Carollia perspicillata*, was investigated. In a food-rewarded flight training procedure, four animals were taught to choose one of two odors simultaneously presented in a three-choice apparatus. *Carollia* chose the odor of ripe banana over that of unripe banana in a spontaneous choice test. With the same method, the bats significantly preferred undiluted banana odor compared to a dilution of 1 : 10, but did not express a preference between undiluted and 1 : 5 dilutions of ripe banana. In a series of reinforced choice tests, the positive stimulus (CS+) was the standardized odor of natural ripe banana. Prominent components of this complex odor were used as CS−, first in monomolecular form and later in combinations (odor cocktails) of increasing similarity to banana odor. In all these tests the bats easily discriminated the natural food odor from the artificial ones. Even a gas chromatographic congruity of 57% between the major components of natural banana odor and an odor cocktail caused no impairment of the olfactory performance. The olfactory discrimination ability of *Carollia perspicillata* supports the assumption that echolocating, frugivorous bats may use their sense of smell to find and select their food.

Key Words—Olfaction, odor discrimination, food odors, bats, *Carollia perspicillata*, Chiroptera, Phyllostomatidae.

INTRODUCTION

Field observations (Fleming and Heithaus, 1986; Rieger and Jakob, 1988) indicate that echolocating, frugivorous bats might use olfactory cues to locate food,

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a supposition supported by histological findings (Bhatnagar and Kallen, 1975) and comparative morphometry of the brain (Frahm, 1985): Among Chiroptera, the nonecholocating, obligatory frugivorous flying foxes and the mainly fruit-eating phyllostomatids show the highest development of olfactory structures. Laska and Schmidt (1986) have shown that the phyllostomatid bat, *Carollia perspicillata*, is able to locate a food source by olfactory cues alone. In the same species, a high absolute sensitivity to food odor components was found (Laska, 1990).

For an efficient trophotaxis by means of olfaction, there are two added requirements. The animal needs (1) a well-developed discrimination ability in order to recognize a specific odor in a usually highly complex olfactory environment, and (2) an ability to detect the concentration gradient emanating from an odor source. The aim of this study was to estimate these two abilities in *Carollia perspicillata*.

METHODS AND MATERIALS

Animals. Four male *Carollia perspicillata* from the colony of the Zoological Institute at the University of Bonn were used for this study. The maintenance of this species has been described by Rasweiler (1977) and Kürten (1983). Food consisted of sliced banana, oat flakes, and honey, supplemented twice a week with protein (Milupa) and water ad libitum.

Test Procedure and Data Analysis. The olfactory discrimination ability of *Carollia* was tested using a food-rewarded training procedure in a three-choice apparatus. It consisted of a starting box, a hexagonal flight arena (1.2 m outer diameter, 0.8 m high) and three boxlike niches (30 × 30 × 20 cm) in adjacent walls (Figure 1A). Each of these contained a landing grid (10 × 15 cm vertical, 10 × 5 cm horizontal), a drawer to present a food reward, and a perforated bottom plate, under which the olfactory stimulus was placed on a shelf (Figure 1B). The whole arena was covered with Plexiglas and was illuminated from above with a weak red light.

In this apparatus the bats were trained to fly from the starting box into the arena when the sliding door was opened. The animals were taught to examine the three options in hovering flight and make a choice by landing on the corresponding grid. After a correct choice, the drawer below the grid was loaded with a small piece of banana ($\approx$ 125 mg) and slid into the arena as a food reward. When landing on a wrong grid, the bat was gently chased away and was allowed to choose again. A test trial ended with the bat returning to the starting box.

Between consecutive trials, the odor-contaminated air was removed with a suction pump (800 liters/min) for 1 min. Twenty seconds after the new arrangement of stimuli, the next trial started with opening the sliding door.

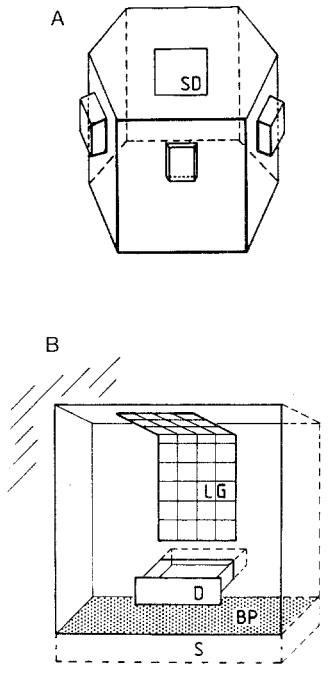


FIG. 1. Schematic drawing of the training apparatus. (A) View of the hexagonal flight arena with the sliding door (SD) separating the starting box, and the three boxlike targets. (B) View from inside the flight arena of one of the three boxlike targets with landing grid (LG), drawer (D) for the food reward, and the perforated bottom plate (BP), under which the odor stimulus, presented in a Petri dish, was placed on a shelf (S).

In all experiments, two olfactory stimuli were presented simultaneously, thus allowing six permutations in a three-choice apparatus. Each of these was presented five times in pseudorandom order, so that 30 trials were analyzed per stimulus combination. In some of the preference tests this program was doubled. The relation of decisions (correct to incorrect in the one-side reinforcement tests, A to B in the preference tests) was examined using the chi-square test, and an error probability of less than 5% taken as the level of significance.

Experiment 1. In the first series of tests both odor alternatives were rewarded, thus allowing the animals to express a spontaneous preference for one of the two. The following comparisons of odor stimuli were tested: (1) ripe vs. unripe banana odor, (2) undiluted vs. 1:5 or 1:10 dilutions of ripe banana odor, and (3) ripe vs. ripe banana odor. The odor stimuli were prepared as follows: Banana odor was standardized by using thawed portions (30 g) of a deep-frozen bunch of mashed ripe fruits (green fruits for the ripeness preference test) sufficient for the whole training program. Banana odor dilutions were

obtained by mixing corresponding masses of thawed banana mash and distilled water to a total mass of 30 g.

Experiment 2. In the second series of tests, only one of the two odor alternatives was rewarded (one-side reinforcement; Stevens, 1975). The positive stimulus (CS+) was always natural banana odor (variety Gros Michel). The following substances, all components of natural banana odor, were used as CS− (the numbers in parentheses correspond to the GC peaks in Figure 2.): (a) isopentyl acetate (9), (b) isobutyl acetate (5), (c) isopropyl acetate, (d) ethyl acetate (2), (e) 2-pentanone, (f) isobutanol (3), (g) *n*-pentyl acetate (10), (h) *n*-butyl acetate (6), and (i) hexyl acetate (11).

Substances a–e were presented as monomolecular odorants. Odor cocktails, i.e., mixtures of odorous substances, consisted of the following combinations: cocktail 2: a + b, cocktail 3: a + b + f, cocktail 5: a + b + f + g + h, cocktail 6: a + b + f + g + h + i.

All CS− were tested in at least three concentrations that were obtained by

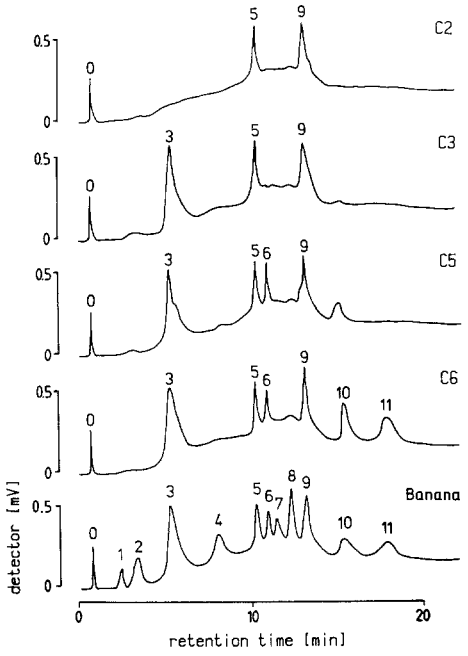


FIG. 2. Gas chromatograms of the odor cocktails used as CS− in the one-side reinforcement tests, and of the standardized odor of natural banana used as CS+. Peak reference numbers: 1, ethanol; 2, ethyl acetate; 3, isobutanol; 4, pentanol; 5, isobutyl acetate; 6, *n*-butyl acetate; 7, ethyl butyrate; 8, pentyl acetate; 9, isopentyl acetate; 10, *n*-pentyl acetate; 11, hexyl acetate.

the solvent dilution method (Dravnieks, 1975): Concentration 1 was identical to the concentration of this substance in the CS+, i.e., banana odor; concentrations 2 and 3 represented 10-fold and 100-fold concentrations, respectively.

In both experiments, all odor stimuli were prepared daily and presented in Petri dishes. For the natural odors, one thawed 30-g portion was used; for the artificial ones, 1 ml of the odorant in the desired concentration was pipetted onto a filter paper that had approximately the diameter of the Petri dish (12 cm). The third option of the three-choice apparatus contained a Petri dish with the pure solvent used for the stimuli (either distilled water or paraffin oil).

Purity, constancy over time, and quantity in terms of concentration values of the odorants used were monitored by gas chromatography. All gas chromatographic analyses were performed using a Carlo Erba Fractovap 4100, using a packed 3-m glass column (OV-101, 5% on Chromosorb W/HP, 80–100 mesh). The flame ionization detector was coupled with a Computing Integrator (Spectra Physics 4100). Isothermic and temperature-programmed head-space analyses were made (probe volume 0.5 ml) using helium as the carrier gas.

RESULTS

Experiment 1: Spontaneous Choice Tests. Three out of four animals significantly preferred the odor of ripe banana to green banana when both alternatives were rewarded (Table 1; animal A: $\chi^2 = 35.2$, $P < 0.001$; animal B: $\chi^2 = 0.06$, $P > 0.5$; animal C: $\chi^2 = 45.0$, $P < 0.001$; animal D: $\chi^2 = 15.0$, $P < 0.001$). In preliminary tests, the odor of unripe fruits proved to be easily detectable for the bats and was chosen as frequently as the odor of ripe banana when there were no odor alternatives. The fact that the preference was shown even in those trials where both alternatives were examined before deciding

TABLE 1. CHOICE BEHAVIOR OF *Carollia perspicillata* IN RIPENESS PREFERENCE TEST, SUBDIVIDED INTO TRIALS IN WHICH BOTH ODOR SOURCES WERE EXAMINED PRIOR TO DECIDING (I) AND WHEN ACCEPTING THE FIRST ODOR SOURCE EXAMINED (II)[a]

	Animal							
	A		B		C		D	
Stimulus	Ripe	Green	Ripe	Green	Ripe	Green	Ripe	Green
I	20	2	2	1	29	1	20	2
II	33	5	29	28	27	3	25	13

[a] Each animal was given 60 trials.

(Table 1), leaves little doubt that *Carollia* really is able to discriminate different stages of ripeness of a fruit by smell alone.

The ability of *Carollia* to respond to intensity differences was estimated testing the undiluted odor of ripe banana against dilutions of 1:10 and 1:5, respectively. Again, preliminary tests with only one odor at a time had shown that the animals respond to the diluted odors as frequently as to the pure one. A dilution of one order of magnitude was significantly discriminated from pure banana odor (animal A: 43:15, $\chi^2 = 13.1$; animal B: 51:9, $\chi^2 = 29.4$; animal C: 50:10, $\chi^2 = 16.6$; animal D: 46:14, $\chi^2 = 17.0$; $P < 0.001$ for all four animals). In the test of pure banana odor vs. 1:5 dilution, the bats failed to express a spontaneous preference (animal A: 15:15; animal B: 14:16; animal C: 17:13; animal D: 16:14). Thus *Carollia* appears able to detect and use concentration differences in choosing a food source.

In a control experiment, two identical odors were presented to the animals simultaneously. The odor sources, both diffusing pure banana odor, were named *X* and *Y* and were presented in the same way as nonidentical stimuli. For all four animals the choices were randomly distributed between the two alternatives (animal A: 15:15; animal B: 14:16; animal C: 16:14; animal D: 16:14). This result demonstrates that (1) the experimenter gave no cues that unintentionally influenced the animals' behavior, and (2) the individual banana odor portions were equally attractive for the bats.

Experiment 2: Reinforced Choice Tests. In a series of one-side reinforcement tests, the bats had to distinguish natural banana odor from monomolecular banana odor components or from odor cocktails of increasing similarity to the food odor. In no case did *Carollia* have any problem solving the discrimination task. The poorest performance of one of the animals in a test was 83% correct choices, a value still significant at the 1% level. Neither the two most prominent esters of banana odor, i.e., isopentyl acetate and isobutyl acetate, nor a substance that is an odor constituent of nearly all fruits, i.e., ethyl acetate, impaired the bats' choice of the reinforced stimulus. Two substances that are preponderantly present in putrefying fruits, isopropyl acetate and 2-pentanon, were also without effect.

Figure 2 shows gas chromatograms of the odor cocktails and of the standardized natural banana odor, and Table 2 the performance of the bats in the corresponding discrimination task. Although the congruity of cocktail 6 (C6) and the prominent components of the food odor is about 57% (C2: 25%; C3: 44%; C5: 52%), even this was not confused with the natural odor by the bats.

DISCUSSION

Apart from a high absolute sensitivity, spatial orientation by olfactory cues implies the ability to discriminate different odor qualities and odor quantities. The data presented here suggest that *Carollia perspicillata* meets these require-

TABLE 2. PERFORMANCE OF *Carollia perspicillata* IN REINFORCED CHOICE TESTS WITH NATURAL BANANA ODOR USED AS CS+ AND ARTIFICIAL ODOR MIXTURES USED AS CS−[a]

	Animal							
	A		B		C		D	
Stimulus	CS+	CS−	CS+	CS−	CS+	CS−	CS+	CS−
Cocktail 2	29	1	28	1	29	1	29	0
Cocktail 3	29	1	28	1	28	2	29	1
Cocktail 5	28	1	26	1	29	1	27	2
Cocktail 6	26	1	27	2	29	0	28	2

[a]Only the data for the tests CS+ vs. CS− in concentration 3 are shown. Each animal was given 30 trials. A sum of decisions for CS+ and CS− lower than 30 indicates that the animal sometimes chose the neutral option.

ments. The ripeness preference test clearly showed that the bats are able to distinguish different stages of ripeness of a fruit. This ability is vital for frugivores as the nutritive value in terms of the percentage of soluble carbohydrates is highly correlated with the state of maturity (Nagy and Shaw, 1980). The anomalous behavior of animal B, which did not exhibit a preference in this test, can be explained as follows: In contrast to its three conspecifics, animal B almost always (in 57 of 60 trials) immediately chose the first fragrant option it examined. With a pseudorandom change in the positions of the two simultaneously presented odor stimuli, this strategy must lead to an equal distribution of choices for the alternatives. Thus we cannot conclude that animal B was unable to solve the discrimination task; rather, there are two other plausible explanations for this behavior: Either the bat "generalized" (Buchholtz, 1973), meaning it recognized the common quality "real banana" in both odors, or it simply learned that in this test situation any odorous option was rewarded. In any case, the animal minimized its energy expenditure to get the food reward, which was identical for both stimuli.

The intensity preference test provides evidence of the basic ability of *Carollia perspicillata* to discriminate between different concentrations of an odorant. For trophotaxis by means of olfaction, this ability is essential in order to recognize the concentration gradient toward an odor source. It must be emphasized that such a spontaneous choice test is not suitable for determining thresholds for the ability to discriminate intensities. The abatement of a spontaneous preference for one of several alternatives only allows conclusions concerning the equality of attractiveness of the stimuli. A one-side reinforcement test with odor alternatives of identical quality, however, would probably have upset and confused the animals and thus impaired the whole training program. The real

efficiency of *Carollia* in detecting intensity differences is most likely far better than the test procedure used here could show. The sudden turns the bats often performed when crossing the odor cloud in front of an option while cruising indicate that even the concentration gradient radiating from a small odor source may be sufficient for intensity differences to be perceived.

The reinforced choice tests demonstrate *Carollia perspicillata* to have a good qualitative discrimination ability for odorants as the bats neither confused the complex natural odor with major constituents of this odor nor with odor mixtures of increasing similarity to the natural stimulus. Further, the concentration of the CS− did not influence the discrimination ability of the animals. Even unphysiological stimulus intensities, such as isopentyl acetate in a concentration 10^4-fold higher than in banana odor, did not result in mistakes, suggesting a high degree of resistance to masking effects.

The responses to monomolecular CS− in concentrations that corresponded closely to those in the CS+ suggest that the bats really did make qualitative discriminations instead of simply comparing intensities. Thus, the olfactory discrimination performance of *Carollia perspicillata* in this study strongly supports the suggestion that echolocating, frugivorous bats may use their sense of smell for foraging.

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FUNGAL ENDOPHYTE-INFECTED GRASSES: ALKALOID ACCUMULATION AND APHID RESPONSE

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Abstract—The occurrence of the alkaloids *N*-formyl and *N*-acetyl loline, peramine, lolitrem B, and ergovaline and the response of aphids to plants containing these compounds were determined in species and cultivars of *Festuca*, *Lolium*, and other grass genera infected with fungal endophytes (*Acremonium* spp., and *Epichloe typhina*). Twenty-nine of 34 host–fungus associations produced one or more of the alkaloids, most frequently peramine or ergovaline. Three alkaloids (lolines, peramine, and ergovaline) were found in tall fescue and in perennial ryegrass infected with *A. coenophialum*, while peramine, lolitrem B, and ergovaline were present in perennial ryegrass and in tall fescue infected with *A. lolii* and in *F. longifolia* infected with *E. typhina*. While *A. coenophialum* and *A. lolii* produced similar patterns of alkaloids regardless of the species or cultivar of grass they infected, isolates of *E. typhina* produced either no alkaloids or only one or two different alkaloids in

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the grasses tested. Aphid bioassays indicated that *Rhopalosiphum padi* and *Schizaphis graminum* did not survive on grasses containing loline alkaloids and that *S. graminum* did not survive on peramine-containing grasses. Ergovaline-containing grasses did not affect either aphid.

Key Words—Endophyte-infected grasses, *Acremonium*, endophytes, *Epichloe typhina*, grass alkaloids, lolines, ergovaline, peramine, lolitrem B, aphid responses, plant resistance to herbivory, *Rhopalosiphum padi*, *Schizaphis graminum*, Homoptera, Aphididae.

INTRODUCTION

Fungal endophytes of grasses, *Acremonium* spp. and *Epichloe typhina* (Pers.) Tul., are distributed worldwide (Latch et al., 1987; White, 1987). *Lolium* and *Festuca* species infected with *Acremonium* spp. endophytes are reported to have enhanced resistance to herbivory (Siegel et al., 1987; Clay, 1989; Dahlman et al., 1990). Resistance to herbivory is but one aspect of the mutualistic symbiotic relationship reported to occur between host and fungus (Siegel et al., 1987; Clay, 1988). Ingestion of endophyte-infected grasses has been shown to be associated with a number of animal toxicoses. Sheep and cattle grazing on tall fescue (*Festuca arundinacea* Schreb.) infected with *A. coenophialum* Morgan-Jones et Gams exhibit fescue toxicity (Stuedemann and Hoveland, 1988), while livestock grazing perennial ryegrass (*Lolium perenne* L.) infected with *A. lolii* Latch, Christensen et Samuels, may suffer from ryegrass staggers toxicity (Mortimer and di Menna, 1985). The closely related endophytes isolated from these and other grasses have been classified as imperfect fungi in the section *Albolanosa* in the genus *Acremonium* (Morgan-Jones and Gams, 1982). The teleomorphic state of *A. typhinum* Morgan-Jones et Gams is *Epichloe typhina* (Morgan-Jones and Gams, 1982), a clavicipitaceous fungus in the tribe Balansieae (Diehl, 1950). *E. typhina* prevents flowering by producing a stroma that surrounds the emerging flower panicle (choke disease). *Acremonium* species and certain biotypes of *E. typhina* spend their entire life cycle growing intercellularly within the grass and are disseminated only through the seed (Siegel, et al., 1987; White, 1988; Clay, 1988).

Endophyte-infected tall fescue and perennial ryegrass contain a number of alkaloids that are active against insect herbivores (Siegel et al., 1987; Dahlman et al., 1990). These include, in infected tall fescue, *N*-formyl and *N*-acetyl loline (Bush et al., 1982), peramine (Fannin et al., 1990), and ergot alkaloids (Yates et al., 1985); in infected perennial ryegrass, they include peramine (Rowan and Gaynor, 1986), lolitrems (Gallagher et al., 1985), and ergot alkaloids (Rowan and Shaw, 1987). Peramine has been reported to be only a feeding deterrent and not directly toxic to Argentine stem weevil (*Listronotus bonar-*

iensis Kuchel). Other of the alkaloids have been reported to be directly toxic to insects. These include lolines (Yates et al., 1989; Dahlman et al., 1990), lolitrems (Prestidge and Gallagher, 1985), and ergopeptine alkaloids, other than ergovaline (Yates et al., 1989). The objective of this study was to determine the distribution of the four types of alkaloids known from endophyte-infected grasses. In addition, the short-term survival of two species of aphids was tested in relation to the occurrence of these alkaloids.

METHODS AND MATERIALS

Sources of Endophytes and Growth of Plants. Infected plants and seed (Table 1) were either donated, collected in Kentucky or New Zealand, or plants were artificially infected with endophytes via meristem wounding of 1-week-old seedlings using the method of Latch and Christensen (1985).

Endophytes, isolated on potato dextrose agar, were identified as *Acremonium* spp. by the presence of single conidia on conidiophores in the typical T configuration (Latch et al., 1984). In addition, all plants were tested prior to alkaloid and aphid analysis by ELISA for the presence of *Acremonium* spp. (Johnson et al., 1985b).

Plants were grown in 10-cm plastic pots in the greenhouse (winter 17–21°C, summer 24–28°C) and maintained in growth condition by dividing older plants and repotting them in a mixture of one part soil (Maury silt loan) and three parts PRO-MIX BR every four to six months. Most, but not all, plants were routinely trimmed to reduce excess foliage. Plants were fertilized biweekly in the winter and weekly in the summer with a dilute solution of soluble fertilizer (317 mg/liter of N–P–K).

Aphid Assay. The oat-birdcherry *Rhopalosiphum padi* L. and greenbug *Schizaphis graminum* Rondani aphids were used to assay for the presence of toxins in endophyte-infected grasses because they were shown previously to be sensitive to endophyte-containing grasses (Johnson et al., 1985a). The aphid cultures were maintained in high numbers by weekly transfers to 1-week-old barley plants (cv. Barsoy). In order to prevent contamination, each aphid species was maintained on plants in screened cages in separate rooms under fluorescent light (50–70 μE/sec/m^2, 14-hr day).

Plant tissue for aphid bioassay consisted of individual test treatment stems (pseudostems containing whorls of leaf sheaths and blades) of tall fescue or multiple stems of perennial ryegrass (three to five stems), fine fescues (six to nine stems) and other grass genera listed in Table 1. The stems were cut from plants at the soil line, stripped of dead tissue, and placed in a 14.8-ml polystyrene cup (containing 11 ml of water and 1 μg/ml of kinetin) by passing the stem(s) through a hole (ca. 2–4 mm diam.) in a waxed paper cap (Figure 1).

TABLE 1. GRASS SPECIES AND CULTIVARS AND FUNGAL ENDOPHYTES USED IN STUDY

Plant code	Infected grass species and cultivars	Fungal endophyte[a]	Origin of endophyte[b]
	Lolium perenne L.		
1	Nui	*A. lolii*	NI
2	Repel	*A. lolii*	NI
3	Ruanui	*A. coenophialum*	AI, *F. arundinacea* (KY 31)
4	Unknown cv.	*E. typhina*	NI
5	Ruanui	*E. typhina*	AI, *F. rubra commutata*[c]
6	Gator	*E. typhina*	AI, *F. rubra commutata*[c]
7	Gator	*E. typhina*	AI, *L. perenne*[d]
8	Gator	*E. typhina*	AI, *F. longifolia*
	Festuca arundinacea Schreb.		
9	G1-320 (Johnstone)	*A. coenophialum*	AI, *F. arundinacea* (KY 31)
10	G1-320 (Johnstone)	*A. coenophialum*	AI, *F. arundinacea* (Au-Triumph)
11	G1-320 (Johnstone)	*A. lolii*	AI, *L. perenne* (Nui)
12	G1-320 (Johnstone)	*A. lolii*	AI, *L. perenne* (Repel)
13	G1-320 (Johnstone)	*A. starrii*	AI, *F. arizonica*
14	G1-320 (Johnstone)	*Phialophora* sp.	AI, *F. arundinacea*[c]
15	G1-320 (Johnstone)	*E. typhina*	AI, *F. longifolia*
16	KY 31	*A. coenophialum*	NI
	F. longifolia Thuill.		
17	SR-3000	*E. typhina*	NI
18	SR-3000	*E. typhina*	AI, *F. rubra commutata*[c]
	F. glauca Vill.		
19	Carolyn blue	*E. typhina*	NI
	F. rubra commutata Guad.		
20	Longfellow	*E. typhina*	NI
21	Enjoy	*E. typhina*	NI
	F. rubra litoralis (Mey.) Auquier		
22	Dawson	*E. typhina*	AI, *F. rubra commutata*[c]
	F. rubra rubra (Gaud.) Hayek		
23	Ensylva	*E. typhina*	NI
24	Pernille	*E. typhina*	NI
25	*F. arizonica* Vasey	*A. heurfanum*	NI
26	*F. paradoxa* Desv.	*Acremonium* sp.	NI
27	*F. versuta* Beal.	*Acremonium* sp.	NI
28	*F. obtusa* Bieler	*A. starrii*	NI
29	*F. gigantea* (L.) Vill.	*Acremonium* sp.	NI
30	*Poa ampla* Merr.	*E. typhina*	NI
31	*Poa autumnalus* (Muhl.) Ell.	*A. coenophialum*	NI
32	*Bromus anomalus* Rupt.	*A. starrii*	NI
33	*Elymus canadensis* L.	*E. typhina*	NI
34	*Sitanion longifolium* Smith	*E. typhina*	NI
35	*Agrostis hiemalis* (Walt.) B.S.P.	*E. typhina*	NI

[a] Plants 4, 7, 17, 18, 22, 35 produced symptoms (stroma) of choke disease in the field.
[b] NI, naturally infected plant; AI, artificially infected strain from indicated plant species (cultivar).
[c] Isolate from unknown cultivar.
[d] Same isolate as in plant 4.

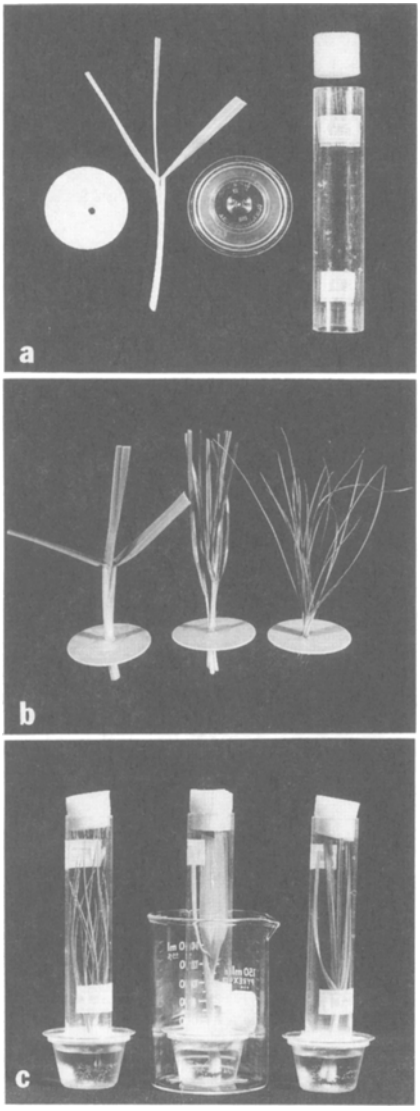


FIG. 1. Stem assay used to determine the presence or absence of chemicals toxic to *Rhopalosiphum padi* or *Schizaphis graminum*. (a) Disassembled components of assay, left to right waxed paper cap with hole to hold stem; cut tall fescue stem; polystyrene cup, Plexiglas cylinder with two fine-mesh screen-covered slits and polyurethane plug at top. (b) Partially assembled components, left to right tall fescue stem, three perennial ryegrass stems, and six *Festuca longifolia* stems. All stems were sealed above and below cap with paraffin. (c) Fully assembled components, left to right stems of *F. longifolia*, tall fescue, and perennial ryegrass. Polystyrene cups contain 11 ml of water and 1 μg/ml of kinetin. Fifty aphids were added to each cage, which was incubated under fluorescent lights for 48–72 hr.

The interface between the paper cap and the stems was sealed with paraffin. The leaves were clipped to accommodate a 11.0 × 2.3-cm Plexiglas cylinder, the diameter of which fitted snugly on the top of the paper cap within the inner rim of the plastic cup. Air exchange in the cylinder occurred through two fine-mesh screen-covered slits in the sides and the polyurethane plug in the top. Fifty apterous (nonwinged) aphids (ca. 3–7 days old) were introduced into the cage by the removal of the plug. The bioassay was run at ambient temperatures under fluorescent light (14 : 10 day–night). There were six test treatment stems for each infected grass species or cultivar. A noninfected control consisting of six stems was present unless otherwise stated. Each sample stem originated from a different infected or noninfected plant used previously for the analysis of alkaloids. The *R. padi* aphids were caged with the stems(s) for 72 hr, while the *S. graminum* aphid test lasted 48 hr. The number of live aphids on the stems and live wandering aphids in the cage were counted. A *t* test, assuming unequal variances, was used to determine if the percent of live aphids on the infected stems was significantly different ($P < 0.05$) from the percent of live aphids on the noninfected stems. After the assay, the stems were tested by ELISA (Johnson et al., 1985b), to verify the presence or absence of endophyte.

Determination of Alkaloids. Harvest of tissue for analysis of alkaloids varied from one to four weeks after the aphid assay. A composite sample of tissue (stems and leaves) from infected plants used in the aphid assay was harvested from three to six plants and cleaned to remove soil debris and dead tissue. The tissue was lyophilized and ground in a Wiley mill to pass through a 40 mesh screen. Samples were stored in a freezer (−20°C) with silica gel. Loline alkaloids were determined, on a 1-g dry wt. sample, by gas chromatography (Kennedy and Bush, 1983).

Peramine was extracted from a 0.1-g sample and the concentration determined by HPLC as described by Tapper et al. (1989).

Lolitrem B was measured by a modification of the procedure of Gallagher et al. (1985). Three-milliliter portions of the lipophilic upper phase from the two-phase extraction used for the peramine HPLC analysis were aspirated through silica columns (Analytichem BondElut, 0.5 g) such that lolitrem B and much of the pigments were retained on the silica. The columns were rinsed with 1 ml of dichloromethane before eluting the lolitrem-containing fraction with 2 ml of dichloromethane–acetonitrile, 4 : 1 v/v. Amounts of lolitrem were estimated by comparison of peak heights with those obtained using a pure sample (gift of Dr. R. Gallagher).

Ergot alkaloids were determined from specific fungi grown in liquid culture and plant tissue. For liquid culture analysis, fungi were isolated and maintained on corn–malt-extract agar and then grown in larger quantities in liquid culture and screened for total alkaloids as described by Bacon (1988).

For plant analyses, ergovaline and its isomer ergovalinine were determined

as an indicator of ergot alkaloid synthesis (Yates et al., 1985; Rowan and Shaw, 1987). Ground samples (0.5 g) were extracted with 5 ml of 80% methanol for 1.5 hr. The mixture was centrifuged at 3000*g* for 15 min and supernatant (2 ml) extracted three times with 10 ml petroleum ether. The petroleum ether was discarded and the extract was diluted with one volume of 80% methanol containing 40 μg/ml of dihydroergocristine methane sulfonate (internal standard). Ergovaline and ergovalinine were quantified by HPLC using a modification of the method of Yates and Powell (1988). The modification involved use of a shorter column, elimination of the silica gel precolumn, and doubling the pump rate. Samples (20 μl) were analyzed on either a Varian 5000 liquid chromatograph using a Phenomenex Ultracarb 5, ODS 30 column (4.6 × 150 mm) and an Upchurch C-130B ground column filled with du Pont Permaphase ODS or a Dynamax microsorb C181 (5 μm) axial compression column (4.6 × 250 mm) with a 4.6 × 15-mm ground module. Fluorescence was monitored on a Kratos Spectroflow 980 programmable fluorescence detector with excitation of 310 nm and detection above a 370-nm long-pass emission filter. Ergovaline and ergovalinine values were combined and the total given as ergovaline.

RESULTS

Alkaloid Production in Endophyte-Infected Grasses. The levels of lolines, peramine, lolitrem, and ergovaline produced singly or in combination varied greatly in the infected grass species and cultivars (Table 2). The most widely distributed alkaloids were peramine and the ergot alkaloid ergovaline. These alkaloids were recovered from the majority of plants infected with *Acremonium* spp. and *E. typhina*. Of the 35 host–fungus combinations studied, peramine was detected in 23 (66%) and ergovaline in 21 (60%). Since ergovaline and other ergot alkaloids are of fungal origin (Bacon, 1988), selected isolates also were grown in liquid culture and analyzed colorimetrically for total ergot alkaloid content. Of the eight isolates grown in artificial media, five produced ergot alkaloids in culture as well as in infected plants. One isolate (from plant 27) did not produce alkaloids in culture or in the infected plants. Two isolates produced ergovaline in the plant (plants 22 and 23) but ergot alkaloids were not found in culture. This lack of synthesis in culture may reflect the age of the isolate used, as continuous artificial culture of isolates apparently results in reduced synthesis of ergot alkaloids (Bacon, 1988).

Loline alkaloids were found in only six (17%) of the host–fungus combinations (plants 3, 9, 10, 16, 29, 31); however, five of these were associated with the five *A. coenophialum*-infected plants examined and the sixth with an unidentified *Acremonium* sp. Not only were lolines identified in naturally infected tall fescue (plants 9, 10, 16) but also in perennial ryegrass (plant 3)

TABLE 2. PRESENCE OF ALKALOIDS AND APHID RESPONSE IN ENDOPHYTE-INFECTED GRASSES

Plant Code[a]	Alkaloid, μg/g dry wt tissue (ppm)[b]				Live aphids on infected stems (%)[d]	
	Lolines	Peramine	Lolitrem B	Ergovaline[c]	*R. padi*	*S. graminum*
1	0	42	10	5.4 (50)	109	4*
2	0	30	5	2.3	93	6*
3	1028	29	0	2.5	25*	1*
4	0	56	0	0	112	1*
5	0	0	0	0	100	111
6	0	0	0	0	98	101
7	0	53	0	0	94	1*
8	0	10	0	1.1	92	8*
9	1800	10	0	2.0	4*	16*
10	1544	4	0	6.2	8*	2*
11	0	4	2	1.1	87	14*
12	0	33	44	1.3	92	9*
13	0	0	0	0	89	112
14	0	0	0	0	102	98
15	0	3	0	0	110	15*
16	1109	10	0	1.5	3*	2*
17	0	22	4	0.9 (38)	100	2*
18	0	0	0	1.8	89	85
19	0	5	0	0.8 (19)	98	13*
20	0	18	0	0.5 (9)	98	1*
21	0	16	0	0	ND[e]	25*
22	0	0	0	0.8 (0)	109	104
23	0	0	0	1.3 (0)	84	67*
24	0	0	0	1.1	ND	100[f]
25	0	0	0	0	ND	104[f]
26	0	132	0	0	ND	15*
27	0	0	2	0 (0)	ND	2[f]
28	0	0	0	0	ND	104[f]
29	2205	0	0	0.7	2*	3*
30	0	32	0	0	101	8[f]
31	972	2	0	0	21*	13*
32	0	8	0	0.3 (29)	ND	10[f]
33	0	12	0	0	ND	ND
34	0	74	0	0.6	ND	1[f]
35	0	16	0	0.4	ND	ND
Number containing alkaloids	6	23	6	20		
% distribution	17	66	17	60		

[a] See Table 1 for identifications of plant code numbers.

[b] Alkaloids were determined from composite tissue samples (3-6 plants) from which stems had been used for aphid assay.

[c] Values for ergovaline include those of its isomer ergovalinine. Numbers in parentheses are for total ergot alkaloids present in liquid culture (mg/liter).

[d] Each assay consisted of 50 aphids each on six test treatment stem(s) of infected or noninfected grasses in screened cages. *Rhopalosliphum padi* (oat birdcherry) aphid, assay for 72 hr. *Schizaphis graminum* (greenburg) aphid, assay for 48 hr; *Significant difference ($P < 0.05$) between the percent live aphids on endophyte-infected stems, as compared to the percent on noninfected stems determined by a t test, assuming unequal variance.

[e] ND, not determined.

[f] No noninfected control available, % alive based on total of 50 aphids.

when artificially infected with the endophyte from tall fescue. Loline alkaloids were not detected in perennial ryegrass infected with its natural endophyte (*A. lolii*) (plants 1 and 2).

Lolitrem B was found in only six host–fungus combinations, four of which were in the four *A. lolii*-infected plants examined. Lolitrem B was present in naturally infected perennial ryegrass (plants 1 and 2) and in tall fescue artificially infected with *A. lolii* (plants 11 and 12). In addition, lolitrem B was recovered from *F. longifolia* infected with *E. typhina* (plant 17) and *F. versuta* (plant 27) infected with an unidentified *Acremonium* sp.

The classes of alkaloids listed in Table 2 were detected singly, in various combinations, or not detected at all (Table 3). Host–fungal associations of *A. coenophialum* in tall fescue cultivars (plants 9, 10, 16) and in one perennial ryegrass cultivar (plant 3) produced peramine, lolines, and ergovaline; *A. lolii* in two perennial ryegrass cultivars (plants 1 and 2) and in two tall fescue cultivars (plants 11 and 12) produced peramine, lolitrem B, and ergovaline. *E. typhina* in *F. longifolia* (plant 17) produced the same alkaloids as found in *A. lolii*-infected plants.

There was a great diversity in the number of different endophyte–host interactions in which only one or two of the alkaloids classes were detected. In eight different plant species–fungal interactions, various combinations of two alkaloid classes were found, while 10 different combinations of fungus and plant

TABLE 3. COMBINATION OF ALKALOIDS IN GRASS–FUNGUS ASSOCIATIONS

Associations (plant code)[a]	Alkaloids[b]			
	Peramine	Lolitrem	Lolines	Ergovaline
No alkaloids detected				
5, 6, 13, 14, 25, 28				
Single alkaloid detected				
4, 7, 15, 21, 26, 30, 33	+			
27		+		
18, 22, 23, 24				+
Two alkaloids detected				
8, 19, 20, 32, 34, 35	+			+
29			+	+
31	+		+	
Three alkaloids detected				
1, 2, 11, 12, 17	+	+		+
3, 9, 10, 16	+		+	+

[a] See Table 1 for identifications of plant code numbers.
[b] +, detection of alkaloid.

produced only one alkaloid. Lolines were always found associated with other alkaloids but never with lolitrem. In six different endophyte-infected grasses, no alkaloids were detected.

Several endophyte taxa were capable of producing similar patterns of alkaloids regardless of the species or cultivar of grass they infected. As previously mentioned, the tall fescue endophyte, *A. coenophialum*, and the perennial ryegrass endophyte, *A. lolii*, produced the same three alkaloids in their naturally infected host grasses as well as when artificially introduced into other grasses. An isolate of *E. typhina* obtained from perennial ryegrass (plant 4) and introduced into a different cultivar (plant 7) produced the same alkaloid, peramine.

However, several associations show that isolates of apparently the same endophyte can produce different alkaloids depending upon the host grass. Of the three *A. starrii*-plant species combinations analyzed, two did not produce any alkaloids in their host grasses (plant 13 artificially infected and plant 28 naturally infected), but the one from *Bromus anomalus* (plant 32) produced two alkaloids in its host. Likewise, three isolates of *E. typhina* from different cultivars of *F. rubra commutata* produced different alkaloid patterns in infected grasses. One of the *E. typhina* isolates, from an unidentified New Zealand cultivar, introduced into two perennial ryegrass cultivars (plants 5 and 6) produced no alkaloids. When this *E. typhina* isolate was introduced into *F. longifolia* (plant 18) and *F. rubra litoralis* (plant 22), ergovaline was present. Unfortunately, the naturally infected host grass was not available for analysis. The other two *E. typhina* endophytes in their own hosts, Longfellow (plant 20) and Enjoy (plant 21), produced peramine in both grasses but in Longfellow ergovaline was identified also.

Lastly, the *E. typhina* from *F. longifolia* (plant 17) was shown to produce peramine, lolitrem, and ergovaline when in its own naturally infected host; only peramine and ergovaline were found when introduced into perennial ryegrass (plant 8), and only peramine was found when introduced into tall fescue (plant 15).

Aphid Response to Endophyte-Infected Grasses. The data in Table 2 indicate that the *R. padi* aphid is only sensitive to those infected plants containing loline alkaloids (plants 3, 9, 10, 16, 29, 31). These plants were all infected with *A. coenophialum*, except for *F. gigantea* (plant 29), which was infected with an unidentified *Acremonium* sp. The specificity of the toxic effect of loline alkaloids is supported by the survival of the *R. padi* aphids on plants that contained no lolines but that have other alkaloids, singly or in various combinations (e.g., plants 1, 4, 17, 30).

The *S. granimum* aphid showed reduced survival on plant material containing peramine only (plants 4, 7, 15, 21, 26, 30) and on plants containing both peramine and loline alkaloids (plants 3, 9, 10, 16, 31). Survival of *S. graminum* aphid did not appear sensitive to ergovaline, as when this alkaloid

occurred by itself (plants 18, 22, 24) the percentage of live aphids on infected and noninfected stems was not significantly different ($P < 0.05$). However plant 23, infected with *E. typhina*, produced ergovaline only and caused a significant ($P < 0.05$) reduction (33%) in the percentage of live aphids. However, since plants 18, 22, and 24 also contained ergovaline only and were not toxic to *S. graminum*, some other factor(s) must be responsible for the effects noted for plant 23. Plant 29 contained both ergovaline and loline alkaloids, and the toxicity of this plant to *S. graminum* may be attributed to the loline alkaloids alone. Lolitrem usually occurred with peramine (plants 1, 2, 11, 12, 17) and consequently it was not possible to determine if the presence this alkaloid alone correlated with aphid toxicity. *S. graminum* did not survive on plant 27, which contained only lolitrem; however, a suitable noninfected control was not available for this combination.

Six plants (plants 5, 6, 13, 14, 25, 28) produced no detectable levels of the four classes of alkaloids and were not toxic to either of the aphids. In this group, plant 14 was infected with a seed-disseminated non-*Acremonium* endophyte, a *Phialophora*-like species (Latch et al., 1984).

DISCUSSION

The concentrations of the alkaloids lolitrem B, lolines, peramine, and ergovaline were analyzed in different grass species and cultivars infected with *Acremonium* spp. and *E. typhina* endophytes. The lolines and lolitrem alkaloids were found in relatively few of the host–fungus combinations and were primarily associated with grasses infected with *A. coenophialum* and *A. lolii*, respectively. The most common alkaloids were peramine and ergovaline, detected in 66% and 60% of the infected grasses, respectively. Various combinations of the four classes of alkaloids were found in 83% of the infected grasses. None of the alkaloids was detected in five host–fungus associations involving different *Acremonium* spp., *E. typhina*, and a *Phialophora*-like sp.

A number of interacting factors are probably responsible for the variations in amounts and numbers of alkaloids present in infected plants. Levels of lolines (Kennedy and Bush, 1983; Belesky et al., 1987) and total ergot alkaloids (Belesky et al., 1988) show seasonal variations. In addition, loline levels in infected plants were increased under greenhouse conditions by repeated cutting of plant tissue (Bush, unpublished data), and levels of lolitrem B and peramine were affected by temperature and plant nutrition (Latch and Tapper, unpublished). In addition, it was assumed that ergovaline and its isomer ergovalinine were the primary ergot alkaloids present in *Acremonium*-infected grasses. However, this is yet to be proven for grasses other than infected tall fescue and perennial ryegrass where they are 40–60% of the total present (Yates et al., 1985; Rowan and Shaw, 1987). Even though the detection method can deter-

mine levels as low as 0.1 ppm, a lack of ergovaline does not completely preclude the presence of other ergot alkaloids. In culture, *Balansia* spp. produced ergot alkaloids that did not contain ergovaline (Bacon et al., 1986).

The biosynthetic origin of the alkaloids (fungal and/or fungal–plant) and the genetics of the symbionts also may affect the concentrations and numbers of alkaloids present. The ergopeptine alkaloids (Bacon, 1988) and peramine (Rowan and Gaynor, 1986) are of fungal origin. Lolitrem B has not been found in culture but paxilline, an obvious biosynthetic intermediate of the lolitrems, has been found when *A. lolii* is cultured and also in infected seeds (Gallagher et al., 1985; Weedon and Mantle, 1987). Lolines have not yet been found in liquid cultures of *A. coenophialum* (Bush, unpublished data). Regardless of how the alkaloids are synthesized, the interaction of symbionts does affect the alkaloids present in the infected plants. Some endophytes produce the same pattern of alkaloids in naturally and in artificially infected grass species and cultivars (i.e., the *A. lolii* and *A. coenophialum* isolates in their own and reciprocal hosts). However, other endophytes do not produce similar patterns of alkaloids in new hosts. This was best illustrated in the case of the *E. typhina* from *F. longifolia* (plant 17). This association produced the same three alkaloids (lolitrem, peramine, and ergovaline) found in perennial ryegrass infected with *A. lolii*. When *E. typhina* from *F. longifolia* was artificially introduced to perennial ryegrass, only peramine and ergovaline were detected, and when inoculated into tall fescue, only peramine. The plants infected with *E. typhina* from *F. rubra commutata* also demonstrated differences in the distribution of peramine and ergovaline. Depending on the source of the endophyte, either no alkaloids were detected, or peramine alone or in combination with ergovaline was found. The dissimilar patterns of alkaloid production may relate to host–fungus compatibility and the eventual survival of the endophyte. The density of mycelium was greater in the naturally infected plant and less in the artificially infected grasses. In addition, approximately 8–12 months after alkaloid analysis it was difficult to find substantial amounts of mycelium in the greenhouse-grown infected perennial ryegrass and tall fescue plants.

Poor performance by two aphid species on endophyte-infected grasses was correlated with the presence of specific alkaloids. Only plants containing lolines, regardless of the presence of peramine and/or ergovaline, affected survival of *R. padi*. Consequently, this aphid species can be used for routine detection of endophytes in those associations known to produce loline alkaloids. Survival of *S. graminum* was reduced by grasses containing a greater number of alkaloids (lolines, peramine, and/or lolitrems). Because of the short duration of the assays, neither chronic toxicity could be studied nor could it be determined if alkaloids in the infected stems were directly toxic or feeding deterants to the aphids.

If these four classes of alkaloids are indeed important in determining herbivory, then the associations that have the largest number of toxic chemicals

should have the greatest spectrum of biological activity. Conversely, endophyte–grass associations lacking these alkaloids should not be biologically active, as was shown in this study. Mammalian or insect toxicity would be further increased if two or more of the alkaloids acted synergistically (Yates et al., 1989). A multiplicity of compounds with differing modes of action would be of great value as it increases the spectrum of activity against insect herbivores as well as reducing the potential for the development of insect resistance to these alkaloids.

Not all grass–endophyte associations confer insect resistance, and increased insect resistance is not the only factor in determining the superiority of infected grasses. Resistance to abiotic stresses, such as drought (Barker et al., 1985; Read and Camp, 1986; Arachevaleta et al., 1989), and improved growth and persistence (Funk et al., 1985; Latch et al., 1985; Hill et al., 1990) has been demonstrated and may play just as important a role in plant survival as does resistance to herbivory. Consequently, all factors need to be considered when plant breeders select endophyte-infected grasses for improved pasture and turf production.

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OLFACTORY-BASED BEHAVIORAL INTERACTIONS AMONG FIVE SPECIES IN THE SOUTHERN PINE BARK BEETLE GROUP[1]

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Abstract—Olfactory-mediated behavioral interactions were investigated among the five scolytid species comprising the southern pine bark beetle group. Behavioral response, as determined from field trap catch data, showed that each species was attracted in greatest numbers to the pheromonal blend produced by conspecifies. Interspecifically, *D. frontalis* displayed no cross-attractancy to *Ips* pheromonal blends, but was weakly attracted to the pheromonal blend of female *D. terebrans*. Three *Ips* species displayed varying degrees of cross-attraction as well as to the *Dendroctonus* pheromonal blends. More specifically, *I. calligraphus* was attracted to *I. avulsus* and, to a very limited extent, also to the male *D. terebrans* pheromonal blend. *I. avulsus* was somewhat more cross-attractive than *I. calligraphus* and showed attraction to the pheromonal blends of female *D. frontalis*, male and female *D. terebrans*, male *I. calligraphus*, and male *I. grandicollis*. *I. grandicollis* showed the greatest degree of cross-attraction, particularly in response to the *Dendroctonus* pheromonal blends.

Key Words—Coleoptera, Scolytidae, bark beetle, *Dendroctonus*, *Ips*, pheromone, olfaction, intraspecific, interspecific, behavior.

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INTRODUCTION

The southern pine bark beetle group consists of five scolytid species, *Dendroctonus frontalis* Zimmermann, *D. terebrans* (Olivier), *Ips calligraphus* (Germar), *I. avulsus* (Eichhoff), and *I. grandicollis* (Eichhoff). All five species coexist in pine forests across southern and southeastern United States. Simultaneous cohabitation of the same host tree by these species usually results in each species occupying a distinct area of the available resource; however, an area occupied by one species generally overlaps with that occupied by another species (Dixon and Payne, 1979; Birch and Svihra, 1979; Birch et al., 1980; Svihra et al., 1980; Paine et al., 1981, Wagner et al., 1985).

Host selection, aggregation, and colonization in these species involves a complex chemical communication system composed of both beetle- and host tree-produced compounds. Electrophysiological investigations have confirmed that individuals of each species possess antennal olfactory receptors capable of detecting semiochemicals produced by conspecifics as well as by members of other species of the group (Payne, 1970, 1971, 1974, 1975; Payne and Dickens, 1976; Dickens and Payne, 1977, 1978; Payne et al. 1982, 1987, 1988; Smith et al., 1988). Previous behavioral investigations have focused on response to selected pheromonal components produced by *D. frontalis* (Renwick and Vité, 1969; Payne et al., 1978, 1988; Richerson and Payne, 1979; Billings, 1985), *D. terebrans* (Siegfried et al., 1986; Payne et al., 1987; Phillips et al., 1989), *I. calligraphus* (Dixon and Payne, 1980; Billings, 1985), *I. avulsus* (Hedden et al., 1976; Richerson and Payne, 1979; Billings, 1985), and *I grandicollis* (Werner, 1972; Dixon and Payne, 1980; Billings, 1985), with and without host tree-produced odors.

More recently, behavioral investigations have concentrated on response patterns of *D. frontalis* (Vité et al., 1964; Svihra et al., 1980; Birch et al., 1980), *D. terebrans* (Vité et al., 1964; Godbee and Franklin, 1976; Phillips et al., 1989), *I. calligraphus, I. avulsus*, and *I. grandicollis* (Vité et al., 1964; Birch et al., 1980; Svihra, 1982) to trees or logs containing the various species of the group. Although these behavioral studies, collectively, were limited to incomplete and/or undefined release rate ratios of beetle-produced pheromonal blends, they have generally confirmed the hypothesis that interspecific responses to olfactory signals do occur, particularly among *D. frontalis, I. calligraphus, I. avulsus*, and *I. grandicollis*, and that these interactions may partially explain the temporal and spatial patterns of host tree colonization as described by Svihra et al. (1980). However, the interspecific, olfactory-based behavioral interactions among these five bark beetle species, as determined by their behavioral responsiveness to natural pheromonal blend components and release rate ratios, and without the influence of host tree odors, have not been investigated.

This paper presents results from field experiments designed to determine the response of members of the bark beetle group to previously reported, naturally occurring pheromonal blends and release rate ratios by the species and to provide insights into the role of specific beetle-produced pheromonal blends in the interspecific aggregation behavior of these five species of the southern pine bark beetle group.

METHODS AND MATERIALS

Experiments were carried out between July and August 1987 on the Raven Ranger District Sam Houston National Forest, east Texas. However, to minimize the potential influence of host tree-produced volatiles on beetle response, the experiments were conducted in a 2-year-old, 40-ha clear-cut. The surrounding forest was predominantly loblolly pine, *Pinus taeda*, and no known bark beetle infestations were present within ca. 83-km radius. The beetle populations were considered to be at endemic levels on a statewide basis.

Test Compounds. Based upon published and unpublished reports of pheromone production and/or release in the five species, pheromonal blends produced by male and female *D. frontalis*, *D. terebrans*, and *I. calligraphus* and by male *I. avulsus* and *I. grandicollis* were individually formulated and tested. A combination of male and female pheromonal blends for *D. frontalis* and *D. terebrans* was also tested. In addition, specific enantiomers of the major pheromonal components were evaluated for *D. frontalis* and the three *Ips* species since previous research had demonstrated differential production and/or responsiveness based upon enantiomeric composition (see references, Table 1). The enantiomeric blends were developed based upon reports of what was found in naturally released volatiles from the species. Neither host odors nor turpentine were included in the above outlined beetle-produced pheromonal blends, because we were interested in the effects of the pheromones alone. As an indication of the occurrence and relative abundance of flying beetles of each species in the test area, a *Dendroctonus* standard, composed of frontalin and turpentine, and an *Ips* standard (Billings, 1985), composed of ipsdienol, ipsenol, and *cis*-verbenol, were included as separate treatments in the experiments. A turpentine-only baited trap and a blank check-trap were included as controls for response to host odor and the trap configuration, respectively.

Elution Devices and Rates. Based upon weight loss per 24 hr, elution devices were developed and rates determined for each compound tested (Table 1). Desired elution rates were obtained by use of glass capillaries and vials of various sizes. Cotton wicks, as well as the distance from the pheromone meniscus to the open end of the capillary or vial also were used to obtain the desired elution rates. Sufficient amounts of each chemical were used to provide the

TABLE 1. EXPERIMENTAL BLENDS OF BEETLE-PRODUCED PHEROMONES

Treatment	Species	Sex[a]	Chemical compounds[b]	Compound ratios	Elution rate (mg/24 hr)	Reference[c]
1	*D. frontalis*	F	(−)-F : (+)-F : TV : V	21.25 : 3.75 : 750 : 6	0.85 : 015 : 30 : 0.24	1,2
2		M	(+)-ENB : (−)-ENB : EXB : V : TV	0.94 : 0.03 : 0.03 : 800 : 9	0.94 : 0.03 : 0.03 : 800 : 9	2,3
3		F&M				
4	*D. terebrans*	F	F : TV : V	1 : 500 : 50	1 : 500 : 50	2,4
5		M	EXB : TV : V	1 : 15 : 1.1	1 : 15 : 1.1	2,4
6		F&M				
7	*I. calligraphus*	M	(*R*)-(−)-,(*S*)-(+)-IPSD : TV : CV	2 : 2.5 : 1	0.2 : 0.25 : 0.1	5,6
8		M	(*R*)-(−)-IPSD : TV : CV	2 : 2.5 : 1	0.2 : 0.25 : 0.1	6
9		M	(*S*)-(+)-IPSD : TV : CV	2 : 2.5 : 1	0.2 : 0.25 : 0.1	6
10		F	TV : CV	5.5 : 1	4.4 : 0.8	5
11	*I. avulsus*	M	(*R*)-(−)-,(*S*)-(+)-IPSD : TV : CV	45 : 1 : 1	0.2 : 0.0044	5,6
12		M	(*R*)-(−)-IPSD : TV : CV	45 : 1 : 1	0.2 : 0.0044	6
13		M	(*S*)-(+)-IPSD : TV : CV	45 : 1 : 1	0.2 : 0.0044	6
14	*I. grandicollis*	M	(*S*)-(−)-,(*R*)-(+)-IPSE : TV : CV	73 : 1 : 1	0.2 : 0.0027 : 0.0027	5,7
15		M	(*S*)-(−)-IPSE : TV : CV	73 : 1 : 1	0.2 : 0.0027 : 0.0027	7
16		M	(*R*)-(+)-IPSE : TV : CV	73 : 1 : 1	0.2 : 0.0027 : 0.0027	7

[a] Sex: F = female; M = male.

[b] CV = *cis*-verbenol (95%); ENB = *endo*-brevicomin (96%; 81.5%, 83%); EXB = *exo*-brevicomin (94%); F = frontalin (98%, 99%, 100%); IPSD = ipsdienol (93%, 94%, 96%); IPSE = ipsenol (99%; 99%, 99%); TV = *trans*-verbenol (99%); V = verbenone (>98%). %s are chemical; optical (+, −) purities, respectively.

[c] 1. Stewart et al. (1977); 2. Payne, West, Silverstein (unpublished); 3. Redlich et al. (1987); 4. Payne et al. (1987); 5. Vité et al. (1972); 6. Vité et al. (1978); 7. Vité et al. (1976).

desired elution rate for a 24-hr period, after which each elution device was recharged to the appropriate level.

Monitoring Traps and Experimental Design. Response of beetles to the different pheromonal blends was monitored with 12-funnel Lindgren traps (Lindgren, 1983). Traps were placed ca. 45 m apart in a single straight row running through the center of the clear-cut. Treatments were initially assigned positions randomly. To minimize positional effects and disproportionate trap catches based upon a nonrandom distribution of beetles, treatments were advanced one position on each subsequent day. Therefore, each treatment occupied each position during the 20-day experiment. Captured beetles were removed every 24 hr and placed in labeled vials for subsequent counting and sexing. Data were analyzed using Fisher's exact test of all possible paired comparisons of cell frequencies (Sokal and Rohlf, 1981).

RESULTS AND DISCUSSION

Response to the *Dendroctonus* and *Ips* standards (treatments 17 and 18) verified that *D. frontalis*, *I. calligraphus*, *I. avulsus*, and *I. grandicollis* were present, but at endemic levels throughout the test area for the duration of the experiment. *D. terebrans* was trapped infrequently and then only in very low numbers.

Host odor, as represented by turpentine alone (treatment 19), did not provide an adequate stimulus to attract any of the scolytid species. It should be emphasized, however, that the release rate used was likely far less than that provided by a stressed tree in nature. In addition, the vertical silhouette alone (treatment 20), as provided by the unbaited trap, did not attract any of the five scolytid species. Therefore, the results verify that response by the beetles to the pheromone-baited traps was in response to the pheromonal blends, coupled with the vertical silhouette provided by the trap itself, and not simply to host odor or to a visual stimulus alone.

Both male and female *D. frontalis* were attracted to the pheromonal blends of female *D. frontalis* and, to a lesser extent, female *D. terebrans* (treatments 1 and 4, Figure 1). This difference in attraction of *D. frontalis* to these pheromonal blends could be attributed to the relatively lower release rate of verbenone and/or the particular frontalin enantiomeric ratio presented in the female *D. frontalis* pheromonal blend. Attraction to both blends could be attributed to the presence of frontalin, *trans*-verbenol and/or verbenone. *D. frontalis* has been reported to be attracted to frontalin, frontalin plus *trans*-verbenol and, to a limited extent, frontalin plus verbenone (Renwick, 1969; Renwick and Vité, 1969, 1970; Payne et al., 1978; Billings, 1985). *trans*-Verbenol has been reported to enhance significantly the attraction of *D. frontalis* to frontalin and frontalin plus

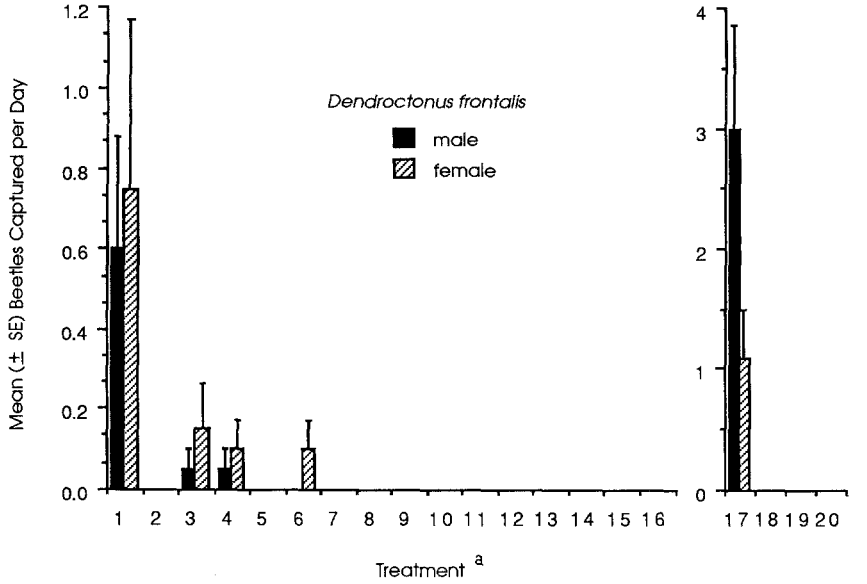


FIG. 1. Mean number of *Dendroctonus frontalis* trapped per day in response to pheromonal compounds eluted at varying rates ($P \leq 0.05$). [a]See Table 1. Vertical bars represent standard errors of means. 1. (−)-F:(+)-F:TV:V; 2. (+)-ENB:(−)-END:EXB:V:TV; 3. 1+2; 4. F:TV:V; 5. EXB:TV:V; 6. 4+5; 7. (*R*)-(−)-, (*S*)-(+)-IPSD:TV:CV; 8. (*R*)-(−)-IPSD:TV:CV; 9. (*S*)-(+)-IPSD:TV:CV; 10. TV:CV; 11. (*R*)-(−)-, (*S*)-(+)-IPSD:TV:CV; 12. (*R*)-(−)-IPSD:TV:CV; 13. (*S*)-(+)-IPSD:TV:CV; 14. (*S*)-(−)-, (*R*)-(+)-IPSE:TV:CV; 15. (*S*)-(−)-IPSE:TV:CV; 16. (*R*)-(+)-IPSE:TV:CV; 17. *Dendroctonus* standard; 18. *Ips* standard; 19. Turpentine standard; 20. Unbaited blank trap.

verbenone (Renwick, 1969; Renwick and Vité, 1969, 1970; Payne et al., 1978). Verbenone, only at very low concentrations, has been reported to enhance the attraction of *D. frontalis* to frontalin plus *trans*-verbenol as well as α-pinene and frontalin plus *trans*-verbenol plus turpentine (Rudinsky, 1973; McCarty et al., 1980).

D. frontalis (Figure 1). Neither sex of *D. frontalis* was attracted to the pheromonal blends of male *D. frontalis* (treatment 2) or male *D. terebrans* (treatment 5). Attraction of *D. frontalis* to the pheromonal blend of the female *D. frontalis* was significantly ($P \leq 0.05$) reduced by the simultaneous presence of the male *D. frontalis* blend (treatment 3). This result can be attributed to (−)-*endo*-brevicomin, *exo*-brevicomin, and/or the relatively high proportion of verbenone eluted from the blends of either the male *D. frontalis* and/or male *D. terebrans*. *endo*-Brevicomin has been reported to reduce *D. frontalis* attraction to frontalin plus α-pinene, with and without *trans*-verbenol or verbenone,

and to frontalin plus turpentine with and without *trans*-verbenol (Vité and Renwick, 1971a; Rudinsky et al., 1974; Payne et al., 1978). More specifically, (−)-*endo*-brevicomin has been shown to reduce *D. frontalis* attraction to frontalure plus turpentine, while (±)-*endo*-brevicomin and (+)-*endo*-brevicomin enhance *D. frontalis* (males more than females) attraction to frontalure plus turpentine (Vité et al., 1985). *exo*-Brevicomin has been reported to reduce *D. frontalis* to frontalin plus α-pinene (Vité and Renwick, 1971a). Furthermore, *endo*-brevicomin plus *exo*-brevicomin also has been reported to reduce *D. frontalis* attraction to frontalin plus *trans*-verbenol and frontalin plus turpentine (Payne et al., 1978). Finally, verbenone, eluted at relatively high rates, reduces *D. frontalis* attraction to frontalin, frontalin plus α-pinene, with and without *trans*-verbenol, and frontalin plus turpentine, with and without *trans*-verbenol (Renwick and Vité, 1969, 1970; Vité and Renwick, 1971a; Rudinsky, 1973; Rudinsky et al., 1974; Payne et al., 1978; McCarty et al., 1980).

Attraction of *D. frontalis* females to the combination of male and female *D. terebrans* pheromonal blends (treatment 6), can be attributed to frontalin and/or the high proportion of *trans*-verbenol eluted from the female *D. terebrans* pheromonal blend. Lack of male *D. frontalis* attraction to the combination of male and female *D. terebrans* pheromonal blends (treatment 6) can likely be attributed to *exo*-brevicomin eluted from the male *D. terebrans* pheromonal blend.

D. frontalis was not attracted to any of the pheromonal blends of the *Ips* species (treatments 7–16). Billings (1985) reported the lack of attraction of *D. frontalis* to *Ips* pheromone, a mixture of 2% *cis*-verbenol, 2% ipsenol, and 2% ipsdienol in a vaseline-based paste, with and without turpentine. Also, interspecific studies, utilizing beetle-infested pine bolts, provided no evidence for the attraction of *D. frontalis* to bolts infested with *I. calligraphus*, *I. avulsus*, or *I. grandicollis* (Vité et al., 1964; Birch and Svihra, 1979; Svihra et al., 1980; Birch et al., 1980; Svihra, 1982).

Too few *D. terebrans* were trapped in the study to analyze its cross-attraction (Figure 2). However, it should be noted that the lack of response of *D. terebrans* to frontalin, frontalure, and *trans*-verbenol, individually, was reported earlier (Payne et al., 1987; Phillips et al., 1989). Payne et al. (1987) reported that a high elution rate of turpentine, in combination with frontalin or frontalure and *trans*-verbenol, was significantly attractive to *D. terebrans*, indicating the potential for interspecific olfactory interactions between *D. terebrans* and *D. frontalis*.

Both male and female *I. calligraphus* were attracted to the pheromonal blends of male *I. calligraphus* and *I. avulsus* containing either racemic ipsdienol (treatments 7 and 11) or (*R*)-(−)-ipsdienol (8 and 12, Figure 3). Significantly ($P \leq 0.05$) more *I. calligraphus* were attracted to the male *I. calligraphus* blend containing racemic ipsdienol (treatment 7) than to the blend containing

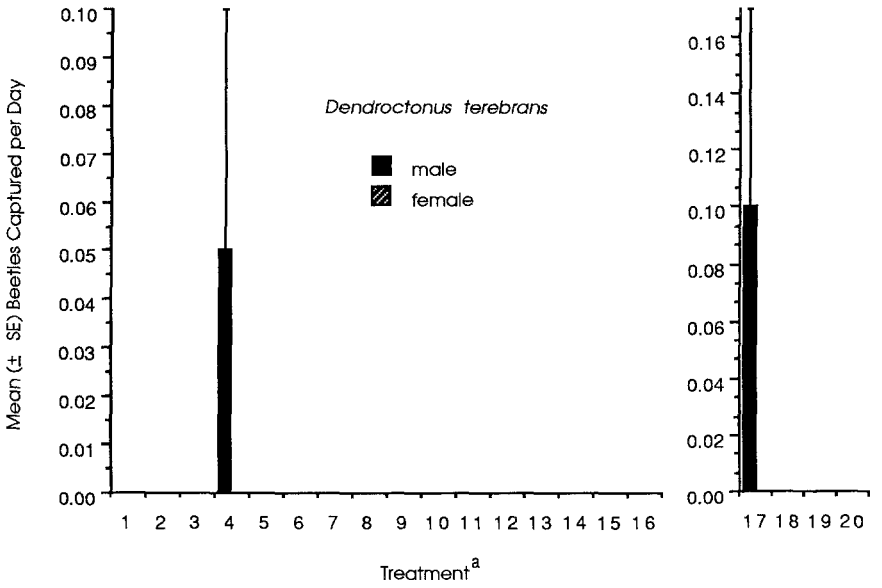


FIG. 2. Mean number of *Dendroctonus terebrans* trapped per day in response to pheromonal compounds eluted at varying rates ($P \leq 0.05$). [a]See Figure 1.

(*R*)-(−)-ipsdienol (treatment 8). However, *I. calligraphus* were not attracted to the blends of male *I. calligraphus* or male *I. avulsus* containing (*S*)-(+)-ipsdienol (treatments 9 and 13). The results support previous reports of the attraction of *I. calligraphus* to ipsdienol plus *cis*-verbenol, and ipsdienol plus *trans*-verbenol (Renwick and Vité, 1972). More specifically, the results are in agreement with Vité et al., (1978), who reported that for *I. calligraphus* (*R*)-(−)-ipsdienol plus *cis*-verbenol was attractive, while (*S*)-(+)-ipsdienol plus *cis*-verbenol was not attractive. However, the results reported here are not consistent with the report by Vité et al. (1978), that (*S*)-(+)-ipsdienol reduced the attraction of *I. calligraphus* to (*R*)-(−)-ipsdienol plus *cis*-verbenol.

I. calligraphus was not attracted to any of the pheromonal blends of male *I. grandicollis* (treatments 14–16). Although there are no previous analyses reported of the behavioral response of *I. calligraphus* to the *I. grandicollis* pheromone, ipsenol, when tested alone, the results presented here can be attributed to the lack of attraction of *I. calligraphus* to ipsenol. Some research has demonstrated the attraction of *I. calligraphus* to *I. grandicollis*-infested bolts (Birch et al., 1980), while other research has shown the lack of such attraction (Vité et al., 1964; Birch et al., 1980; Svihra, 1982). Furthermore, *I. grandicollis*-infested bolts have been shown in some instances to enhance *I. calligraphus* attraction to *I. calligraphus*-infested bolts and in other instances to reduce

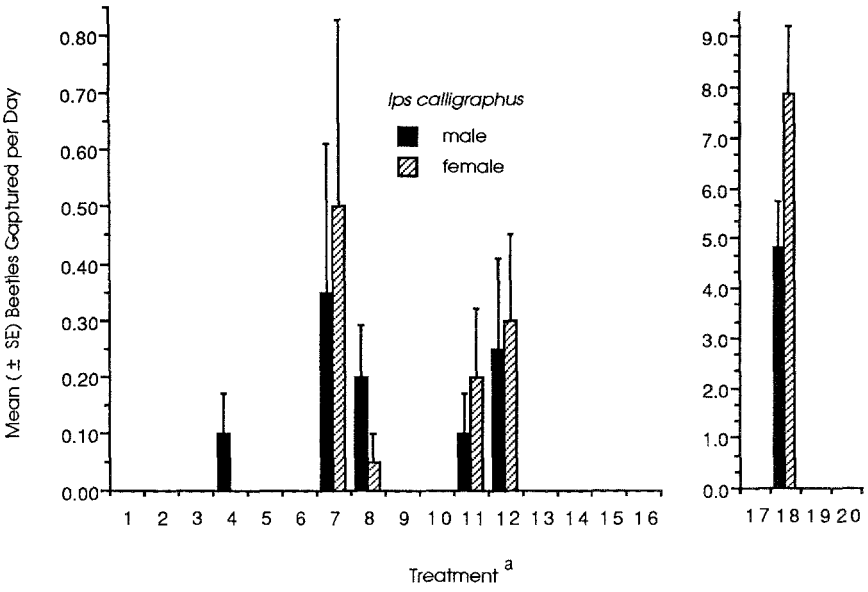


FIG. 3. Mean number of *Ips calligraphus* trapped per day in response to pheromonal compounds eluted at varying rates ($P \leq 0.05$). [a]See Figure 1.

such attraction (Birch et al., 1980). Such mixed results may be due to experimental procedure and, therefore, may warrant reexamination of this phenomenon.

I. calligraphus also was not attracted to any of the *D. frontalis* blends (treatments 1–3), or to virtually any of the *D. terebrans* blends (treatments 4–6). Dixon and Payne (1980) similarly reported the lack of attraction of *I. calligraphus* to frontalin, *trans*-verbenol, *endo*-brevicomin, *exo*-brevicomin, verbenone, and turpentine, when tested individually or in various combinations. *I. calligraphus* was reported to be unresponsive to *D. frontalis*-infested bolts (Vité et al., 1964; Birch et al., 1980; Svihra, 1982). However, Dixon and Payne (1980) found that *I. calligraphus* was attracted to bolts infested with female *D. frontalis*.

Male and/or female *I. avulsus* were attracted to the pheromonal blends of male *I. calligraphus* (treatments 7–9) and male *I. avulsus* (treatments 11–13, Figure 4). Significantly ($P \leq 0.05$) more *I. aavulsus* were attracted to the blends of male *I. calligraphus* containing racemic ipsdienol or (*R*)-(−)-ipsdienol (treatments 7 and 8) than to the blend containing (*S*)-(+)-ipsdienol (treatment 9). *I. avulsus* were also significantly ($P \leq 0.05$) more attracted to the blend of male *I. avulsus* containing (*R*)-(−)-ipsdienol (treatment 12) than to the blend containing racemic ipsdienol (treatment 11). Significantly ($P \leq$

0.05) more female than male *I. avulsus* were attracted to the blend of male *I. avulsus* containing (*R*)-(−)-ipsdienol (treatment 12). The results are in agreement with those reported for the attraction of *I. avulsus* to racemic ipsdienol and (*R*)-(−)ipsdienol plus (*S*)-(−)-ipsenol (Hedden et al., 1976; Vité et al., 1978), and the attraction of *I. avulsus* to pine bolts infested with either male *I. avulsus* or male *I. calligraphus* (Vité et al., 1964; Birch et al., 1980; Svihra et al., 1980; Svihra, 1982).

I. avulsus showed little or no attraction to male *I. grandicollis* pheromonal blends (treatments 14–16), with only a few male *I. avulsus* attracted to the *I. grandicollis* pheromonal blend containing racemic ipsenol (treatment 14). Hedden et al. (1976) reported attraction of *I. avulsus* (males more than females) to ipsenol. Birch et al. (1980) also reported attraction of *I. avulsus* to male *I. grandicollis*-infested pine bolts. *I. avulsus* displayed some, although not significant, attraction to female and male plus female *D. frontalis* and *D. terebrans* blends (treatments 1 and 4–6). While some studies have reported the lack of attraction of *I. avulsus* to *D. frontalis*-infested bolts (Birch et al., 1980; Svihra, 1982), others have demonstrated attraction (Vité et al., 1964; Svihra et al., 1980).

Both male and female *I. grandicollis* were attracted to the pheromonal blend of male *I. grandicollis* containing (*S*)-(−)-ipsenol (treatment 15, Figure

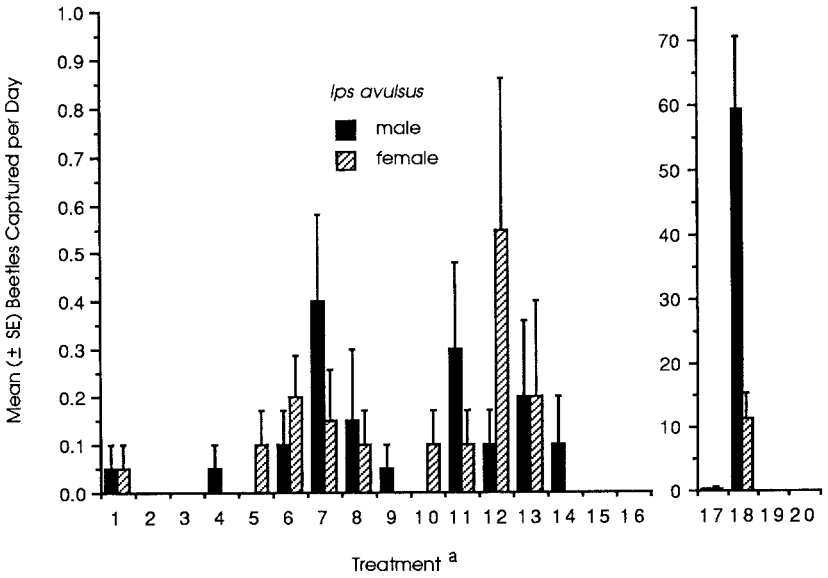


FIG. 4. Mean number of *Ips avulsus* trapped per day in response to pheromonal compounds eluted at varying rates ($P \leq 0.05$). [a]See Figure 1.

5). The pheromone accounts, at least in part, for the attraction of *I. grandicollis* to male *I. grandicollis*-infested bolts reported earlier (Vité et al., 1964; Vité and Renwick, 1971b; Birch and Svihra, 1979; Birch et al., 1980; Svihra et al., 1980; Svihra, 1982). (*R*)-(+)-ipsenol was not attractive by itself (treatment 16), and significantly ($P \leq 0.05$) reduced attraction of *I. grandicollis* to the male *I. grandicollis* blend containing (*S*)-(−)-ipsenol (treatment 14). The results agree with previous reports that (*R*)-(+)-ipsenol significantly ($P \leq 0.05$) reduced the attraction of *I. grandicollis* to (*S*)-(−)-ipsenol (Vité et al., 1976). Significantly ($P \leq 0.05$) more *I. grandicollis* females than males conspecifics were attracted to the blends of male *I. grandicollis* containing (*S*)-(−)-ipsenol (treatment 15). The results support earlier reports that ipsenol (Vité and Renwick, 1971b; Vité et al., 1976), and more specifically (*S*)-(−)-ipsenol (Vité et al., 1976) are attractive to *I. grandicollis*.

I. grandicollis was attracted to the blends of *I. calligraphus* (treatments 7, 9, 10) and *I. avulsus* (treatments 11 and 12), but at a low level. Vité et al. (1976) reported the lack of attraction of *I. grandicollis* to ipsdienol. However, while some research has shown the attraction of *I. grandicollis* to bolts infested with *I. calligraphus* (Vité et al., 1964; Birch et al., 1980; Svihra, 1982) and *I. avulsus* (Birch et al., 1980), others have reported a lack of attraction (Vité et al., 1964; Birch et al., 1980; Svihra, 1982).

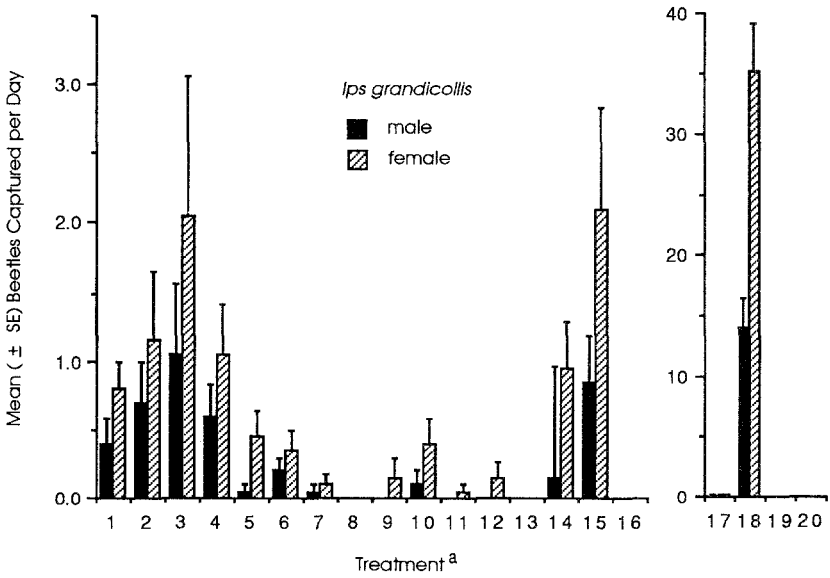


FIG. 5. Mean number of *Ips grandicollis* trapped per day in response to pheromonal compounds eluted at varying rates ($P \leq 0.05$). [a]See Figure 1.

I. grandicollis was attracted to the blends of male and female *D. frontalis* (treatments 1–3) and *D. terebrans* (treatments 4–6). Significantly ($P \leq 0.05$) more *I. grandicollis* were attracted to a combination of male and female *D. frontalis* blends (treatment 3) than to either blend alone (treatments 1 and 2). In contrast, significantly ($P \leq 0.05$) more *I. grandicollis* were attracted to the blend of female *D. terebrans* (treatment 4) than to the blends of male *D. terebrans* (treatment 5) or a combination of male plus female *D. terebrans* (treatment 6). This general attraction of *I. grandicollis* to the pheromonal blends of both *Dendroctonus* species supports previous reports of the attraction of *I. grandicollis* to frontalin plus *trans*-verbenol (Dixon and Payne, 1980), as well as to *D. frontalis*-infested bolts (Vité et al., 1964; Birch and Svihra, 1979; Dixon and Payne, 1980; Birch et al., 1980; Svihra et al., 1980).

D. frontalis, the primary pioneering species of this group (Birch and Svihra, 1979; Svihra et al., 1980; Birch et al. 1980), apparently does not utilize *Ips* pheromones in host selection. Cross-attractancy of *I. calligraphus* to male *I. avulsus* and of *I. avulsus* to male *I. calligraphus* was verified. However, based upon the results here and the mixed results of the other cited reports, the attraction of *I. calligraphus* and *I. avulsus* to *I. grandicollis* or *D. frontalis* still remains in question. *I. grandicollis* was the least specific in its responsiveness, displaying the greatest degree of cross-attractancy among the cohabiting species. Based upon the results here, as well as the other cited reports, the attraction of *I. grandicollis* to *D. frontalis*, *D. terebrans*, *I. calligraphus*, and *I. avulsus* is apparent and may facilitate its ability to locate and occupy the wide range of host conditions used by the other species.

Although cross-attraction ranged from none for *D. frontalis* in response to the pheromonal blends of *Ips*, to extensive for *I. grandicollis*, it is probable that members of all five species benefit from the olfactory-based behavioral interactions. The interactions are likely most important when population levels are endemic, particularly for the less aggressive species, i.e., *I. grandicollis*, when the joint efforts of the group members are essential for overtaking the defenses of the host trees. Certainly this would increase the probability of the successful colonization of a resistant host by each species. The low numbers of beetles trapped during the study most likely were due, in part, to the fact that population levels of the species were endemic. However, it is also likely that the absence of host volatiles in the treatments made them less attractive. It is well known that host volatiles enhance the response of beetles to pheromones.

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DEFENSIVE ROLE OF TROPICAL TREE RESINS: ANTITERMITIC SESQUITERPENES FROM SOUTHEAST ASIAN DIPTEROCARPACEAE

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Abstract—Chemical components demonstrating antiinsectan properties were isolated and identified from crude resins of four *Dipterocarpus* species. The most active compounds against Southeast Asian termites (*Neotermes* spp.) proved to be alloaromadendrene, humulene, and caryophyllene. These results suggest that these sesquiterpenes, which occur in many dipterocarps, play an important role in defense against insects.

Key Words—Dipterocarpaceae, Isoptera, termites, insecticides, sesquiterpenes, alloaromadendrene, humulene, caryophyllene, tropical biology.

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[7]For the purposes of this discussion the possible New World extension of the Dipterocarpaceae, *Pakaraimaea*, is not considered (Maguire et al., 1977; Kostermans, 1985, 1989).

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INTRODUCTION

Often complex mixtures, wood resins are thought to be important defensive agents (Langenheim, 1990). In temperate regions, resins of *Pinus* form part of a strategy to resist biological attack (Berryman, 1972). These resins, whose production can be enhanced by a variety of physical and chemical insults (Cheniclet, 1987; Miller et al., 1986), possess a number of biologically active natural products. α-Pinene, for example, is well known for its antimicrobial and insecticidal properties (Bridges, 1987).

Among tropical trees, the Neotropical legume genus *Hymenaea* produces chemically complex leaf and wood resins (Martin et al., 1972). Several investigations indicate that resins of *Hymenaea coubaril* L. protect these trees against biological attack. These resins are toxic to the herbivorous lepidopteran *Spodoptera exigua* (Stubblebine and Langenheim, 1977) and the fungus-growing ants *Atta cephalotes* (Howard et al., 1988), repellent to *A. cephalotes*, and inhibit growth of ant-associated and other fungi (Langenheim et al., 1980; Hubbell et al., 1983). The sesquiterpene caryophyllene oxide is toxic to ants and inhibits growth of ant-associated fungi (Howard et al., 1988).

Wood resins of the paleotropical plant family Dipterocarpaceae contain a variety of terpenoids (Bisset et al., 1966, 1967, 1971; Diaz et al., 1966). While the structures of a number of these compounds are known, the role of these terpenes in the natural setting is not yet well understood.

Some of these chemicals may function in defense. Crude resins and purified resin sesquiterpenoids from *Dipterocarpus kerrii* King killed termites and inhibited fungal growth (Richardson et al., 1989). Volatile components of crude resins of *Hopea papuana* Diels also inhibit fungal growth (Messer, 1985), as do polyphenols extracted from bark of dipterocarps (Geewananda et al., 1986; Sultanbawa et al., 1987; Bokel et al., 1988). Dipterocarp timbers themselves have been shown resistant to biological attack (Sen-Sarma, 1963; Bakshi et al., 1967; Sen-Sarma and Chatterjee, 1968; Balasundaran and Gnanharan, 1986).

We undertook a survey of dipterocarp resins to determine if the biological activity and presumed defensive roles reported earlier (Richardson et al., 1989) represented a more general phenomenon. The idea was to identify biologically active resins and to isolate and characterize chemicals underlying any observed biological activity.

MATERIALS AND METHODS

Tree Resins

Dipterocarp resins were collected from man-made trunk wounds cut into trees growing in experimental forests, forest preserves, or timber concession

areas in Sumatera, Kalimantan, and Java. Typically, at breast height, a 15 × 1-cm diagonal slash was cut through the bark to the wood. Trees were later checked for resin exudation at irregular intervals, and fresh resins scraped into glass jars with stainless-steel spatulas. If resin samples could not be immediately brought to the laboratory, approximately 10% v/v dichloromethane was added to the sample to maintain it in the liquid state. Prior to laboratory procedures, dichloromethane was evaporated from such samples by allowing the jar to stand overnight at ambient temperature. The resin collection protocol did not harm trees in any visible manner.

Wounds were reopened as necessary to maintain resin flow. In some cases 2-chloroethylphosponic acid was applied to trunk wounds to stimulate resin exudation (Messer, 1990).

Termite Bioassays

Toxicity of crude resins, fractions, and pure chemical standards was evaluated in no-choice bioassays with *Neotermes ?dalbergiae* (Kalshoven) (S. Bacchus, personal communication) obtained from commercial sources in Bogor, Indonesia. This termite species was chosen because, although it does attack some live tree species (Roonwal, 1970), it does not normally attack living Dipterocarpaceae trees. Thus *N. ?dalbergiae* might be more susceptible to any defensive chemicals, because it would not have evolved resistance mechanisms. Dichloromethane solutions of test materials were applied to 4.5-cm Whatman No. 1 filter paper disks. After the solvent had evaporated at ambient temperature, the filter papers were placed in 5-cm glass Petri dishes and 25 worker termites added. This quantity was chosen because it represented the largest number of termites that could be counted conveniently. Except where noted, each sample was tested in eight replicate dishes, so that 200 animals were used at the beginning of each bioassay. Each day the number of termites surviving was recorded, dead termites were removed, and the filter papers were moistened with distilled water. Dishes were kept covered in a darkened room at ambient temperature (25–31°C) and humidity. The average relative humidity in Bogor is 85%.

Statistical Testing and Results

Where appropriate, bioassay results were subjected to one-way analysis of variance (ANOVA). The mean number of termites remaining in each treatment group on a given day was used as a single observation for the ANOVA. Data were examined for normality prior to statistical testing, which was conducted with Data Desk (Velleman, 1989). To facilitate direct comparison of bioassays conducted at different times, results are normalized to the control (filter paper treated with dichloromethane) experiment conducted for each bioassay.

Termite Hindgut Symbionts

For studies of the effect of resins or fractions on termite hindgut symbionts, fifty *N. ?dalbergiae* were placed in a Petri dish prepared for bioassay as described above. Filter papers were treated with 12.5-mg or 25-mg quantities of resins. At daily intervals, hindgut contents of five termites were examined (Messer and Lee, 1989). The relative abundance of each protozoan morphotype was scored on a subjective scale from 0 to 3 (absence to presence), and the means for each morphotype were added to give an estimate of the symbiont populations at 24-, 48-, and 72-hr time points.

Hyperbaric oxygen treatments were used to defaunate termites (Messer and Lee, 1989). Termites were placed in a Plexiglas pressure vessel and exposed to 100% oxygen at 300 kPa for 75 minutes. When large numbers (>100) of termites were treated, oxygen was allowed to flow through the pressure vessel at a rate of approximately 25 ml/min. This prevented oxygen tension from dropping, as a result of normal metabolic activity, to levels not lethal to hindgut protozoa.

Fractionation and Isolation

Resins were fractionated into nonpolar and polar fractions by flash chromatography using 2.5 g of crude resin with a 5-cm bed of silica gel (90–230 mesh) in a 35-mm-ID column. The nonpolar and polar fractions were obtained by eluting with 120 ml of *n*-pentane and 120 ml of dichloromethane, respectively.

Nonpolar fractions were further fractionated with *n*-pentane and a 15-cm bed of silica gel. In order to obtain good fractionation, it was necessary to refrigerate the column and solvents to 4–8°C. The refrigeration was needed because of the high ambient temperatures in Indonesian laboratories and the low polarity of many of these compounds. Fractions were analyzed by thin-layer chromatography (silica gel) eluting with 100% hexane and visualizing with anisaldehyde (Richardson et al., 1989).

Other Analytical Methods

Analytical gas chromatography (GC) was performed in Indonesia with a Shimadzu GC-7A instrument equipped with a 3-m × 2-mm glass column packed with 3% OV-101 on 80/100-mesh GasChromQ, and a flame ionization detector connected to a Hewlett-Packard 3390 integrating chart recorder. Isothermal elutions were performed with nitrogen at 175°C.

Individual chromatographic fractions were analyzed on a Perkin-Elmer Sigma 3B gas chromatograph using a Quadrex 25-m × 1-μm methylsilicone

capillary column (isothermal at 130°C). Mass spectra (MS) and GC-MS (EI and CI) data were obtained using a Finnigan model 4500 GC-MS system. High-pressure liquid chromatographic separations were carried out on an Eldex model 9600 HPLC using a BarSpec Chrom-A-Scope detector and a Supelco 25-cm × 10-mm Supelcosil LC-18 column (isocratic using 100% CH_3CN at 2.5 ml/min). Proton NMR spectra were obtained in $CDCl_3$ solutions on a Varian model XL 400 (400 MHz) NMR spectrometer. Since useful high-field [^{1}H]NMR data for the sesquiterpenes we found do not appear to be available in the literature, we present their spectra below [except for the case of caryophyllene, whose spectrum is complicated because of slowly interconverting conformers (Warnhoff and Srinivasan, 1973; Schirchama et al., 1981)].

Authentic samples of (−)-alloaromadendrene, (+)-calarene, (−)-caryophyllene, (−)-copaene, (−)-α-gurjunene, (+)-γ-gurjunene, and humulene were obtained from Fluka Chemical Corp.; (−)-caryophyllene oxide was obtained from Aldrich Chemical Co. Optical rotations were measured using a Perkin-Elmer 241 polarimeter.

[^{1}*H*]*NMR Spectral Data*

Alloaromadendrene. δ 0.22 ppm (dd, 1H, J = 10.9, 9.4 Hz), 0.52 (ddd, 1H, J = 10.9, 9.4, 6.2), 0.92 (d, 3H, J = 6.8), 0.94 (s, 3H), 0.98 (s, 3H), 1.14 (dddd, 1H, J = 14.2, 11.1, 9.5, 7.1), 1.3 (m, 1H), 1.65–1.76 (m, 2H), 1.77–1.90 (m, 3H), 2.05 (m, 1H), 2.26 (ddd, 1H, J = 13.4, 6.7, 4.0), 2.32 (ddd, 1H, J = 13.7, 9.5, 6.6), 2.65 (q, 1H, J = 8.1), 4.69 (t, 1H, J = 2.1), 4.71 (br s, 1H).

Calarene. δ 0.54 (d, 1H, J = 9.2), 0.72 (td, 1H, J = 9.5, 3.5), 0.95 (d, 3H, J = 6.9), 0.96 (s, 3H), 0.99 (s, 3H), 1.05 (s, 3H), 1.32–1.43 (m, 3H), 1.71 (m, 1H), 1.72 (ddd, 1H, J = 13.1, 5.9, 1.6), 1.87–2.00 (m, 3H), 2.19 (m, 1H), 5.22 (dt, 1H, J = 4.5, 2.2).

Caryophyllene Oxide. δ 0.94 (m, 1H), 0.97 (s, 3H), 0.99 (s, 3H), 1.18 (s, 3H), 1.30 (dddd, 1H, J = 12.5, 10.6, 8.1, 4.4), 1.40 (dddd, 1H, J = 14.8, 13.5, 10.5, 4.3), 1.59 (t, 1H, J = 10.5), 1.62 (dddd, 1H, J = 14.8, 4.9, 3.3, 1.3), 1.66 (ddd, 1H, J = 10.6, 8.3, 0.6), 1.74 (t, 1H, J = 10.0), 2.04–2.13 (m, 2H), 2.23 (ddd, 1H, J = 16.5, 8.1, 4.1), 2.32 (ddd, 1H, J = 13.1, 7.8, 4.5), 2.60 (q, 1H, J = 9.4), 2.86 (dd, 1H, J = 10.6, 4.3), 4.83 (d, 1H, J = 1.7), 4.95 (d, 1H, J = 1.8).

Copaene. δ 0.76 (s, 3H), 0.81 (d, 3H, J = 6.2), 0.83 (d, 3H, J = 6.4), 1.44–1.58 (m, 6H), 1.64 (m, 1H), 1.64 (d, 3H, J = 2.0), 1.70 (dd, 1H, J = 10.9, 7.8), 2.07 (dtd, 1H, J = 6.3, 3.1, 1.3), 2.16 (m, 2H), 5.18 (o, 1H, J = 1.5).

α-Gurjunene. δ 0.76 (m, 1H), 0.82 (s, 3H), 0.89 (d, 3H, J = 7.1), 1.04

(br d, 1H, J = 9.6), 1.09 (s, 3H), 1.31 (m, 2H), 1.44 (dt, 1H, J = 12.2, 9.0), 1.63 (dd, 3H, J = 2.3, 1.2), 1.70 (m, 1H), 1.73 (m, 1H), 1.79 (m, 1H), 1.86 (m, 1H), 2.09 (m, 1H), 2.23 (m, 1H), 2.71 (m, 1H).

γ-Gurgunene. δ 0.81 (d, 3H, J = 7.0), 0.98 (d, 3H, J = 7.0), 1.19 (dt, 1H, J = 11.3, 6.9), 1.36 (dddd, 1H, J = 13.5, 8.9, 4.3, 2.8), 1.53 (dd, 1H, J = 12.4, 6.3), 1.63 (m, 1H), 1.65 (m, 1H), 1.67 (m, 1H), 1.69 (s, 3H), 1.74 (m, 1H), 1.76 (m, 1H), 1.86 (m, 1H), 2.53 (br q, 1H, J = 6.9), 2.79 (m, 1H), 2.87 (m, 1H), 4.72 (br s, 1H), 4.73 (br s, 1H), 5.29 (dt, 1H, J = 5.7, 2.8).

Humulene. δ 1.04 (s, 6H), 1.41 (d, 3H, J = 1.6), 1.61 (d, 3H, J = 1.6), 1.89 (d, 2H, J = 7.8), 2.06 (m, 2H), 2.08 (m, 2H), 2.49 (d, 2H, J = 7.3), 4.85 (td, 1H, J = 7.5, 2.0), 4.93 (td, 1H, J = 6.8, 1.6), 5.13 (d, 1H, J = 15.9), 5.58 (dt, 1H, J = 16.2, 7.2).

RESULTS AND DISCUSSION

Toxicity of Dipterocarp Resins

Initial bioassays conducted with applications of 25 mg of crude resin applied to filter papers indicated that the resins of dipterocarp species vary in their toxicity to *N. ?dalbergiae* (Figures 1–6). In some experiments, termites

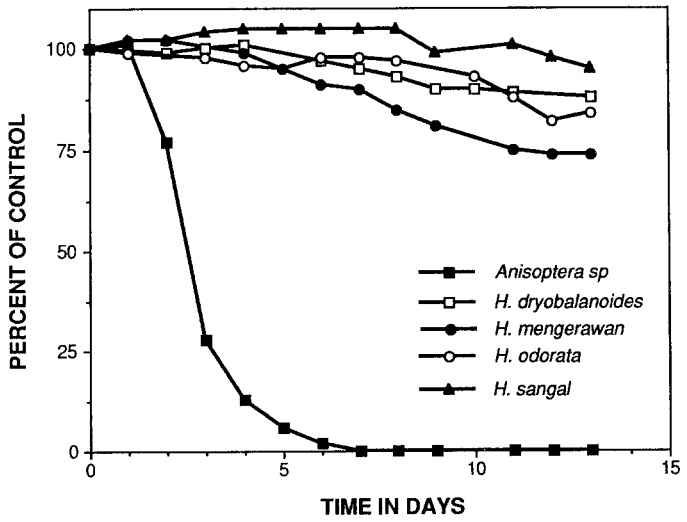


FIG. 1. Toxicity of crude trunk resins of several genera of Dipterocarpaceae to *Neotermes ?dalbergiae* drywood termites. To enable comparisons of several bioassays, results are normalized to controls for each experiment. In some cases control groups died more rapidly than treatment groups, making some values greater than 100%. Details of bioassay procedures are reported in Methods and Materials.. Toxicity of an undetermined *Anisoptera* sp. resin, and *Hopea* spp. resins.

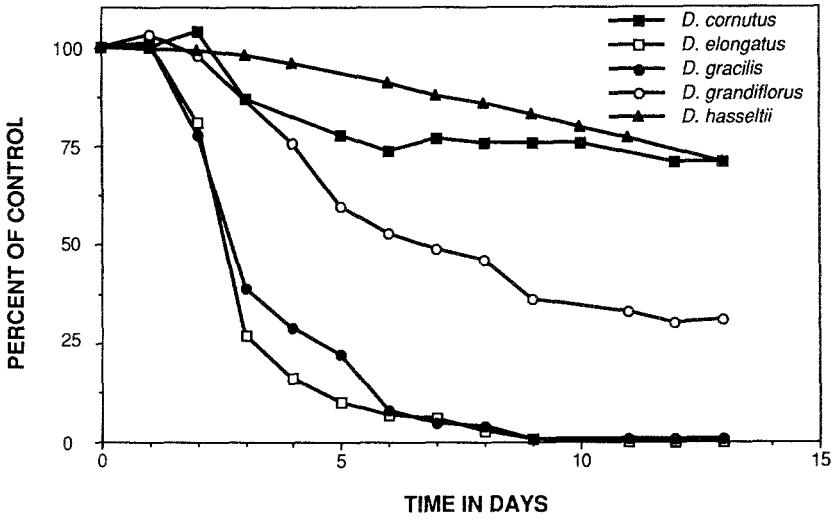


FIG. 2. Toxicity of *Dipterocarpus* spp. resins. See Figure 1 legend.

in the control dishes died at a slightly greater rate than did termites in the experimental dishes. Transformation of experimental results from these bioassays gave values of greater than 100%.

Under the bioassay conditions used, resins from the genera *Dipterocarpus* and *Anisoptera* were apparently the most toxic (Figures 1–3). It is interesting

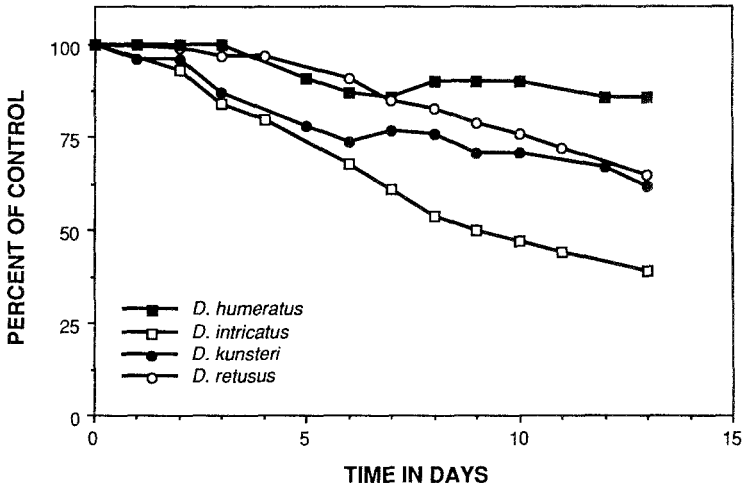


FIG. 3. Toxicity of *Dipterocarpus* spp. resins. See Figure 1 legend.

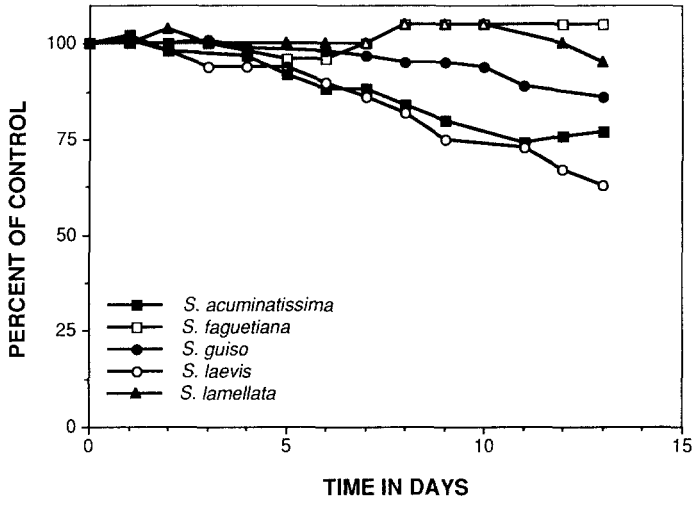


FIG. 4. Toxicity of *Shorea* spp. resins. See Figure 1 legend.

to note that these resins are the only ones studied that persist in a liquid state on the slashed tree trunk. Resins of *D. retusus*, *D. intricatus*, *D. hasseltii*, and *D. grandiflorus* were selected for further study because they displayed a broad range of toxicity and were available in large (>25 g) quantities.

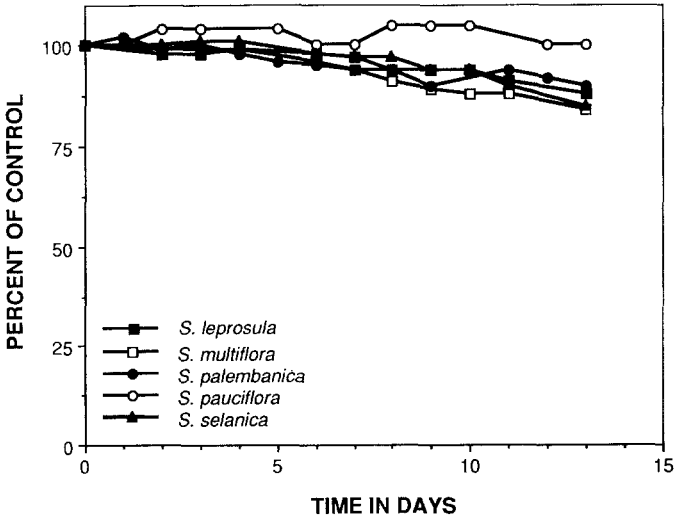


FIG. 5. Toxicity of *Shorea* spp. resins. See Figure 1 legend.

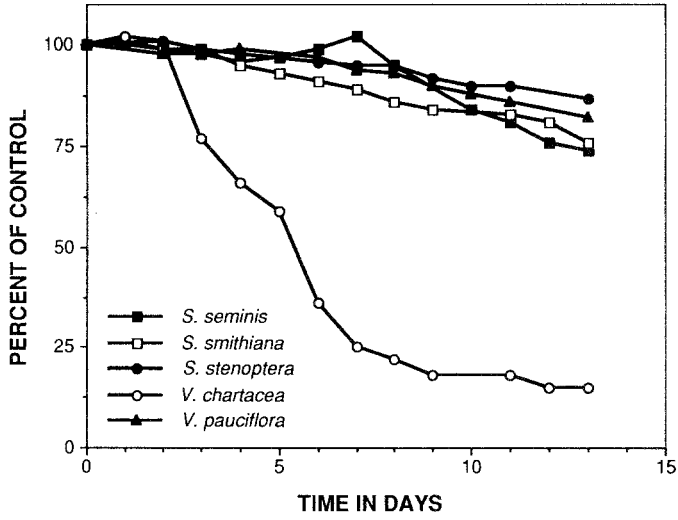


FIG. 6. Toxicity of *Shorea* spp. and *Vatica* spp. resins. See Figure 1 legend.

Fractionation of Active Components

Bioassays on nonpolar and polar resin fractions showed that the toxic components were contained only in the nonpolar fractions (Figure 7). These nonpolar resin fractions were further purified by flash chromatography with cold *n*-pentane, and these fractions were tested for termicidal activity.

The major components of the toxic fractions were isolated by HPLC and characterized by [^{1}H]NMR and GC-MS data. These components were determined to be the sesquiterpenes shown in Figure 8, and their identities were verified by direct comparisons with authentic samples. Capillary GC analyses of crude resins showed these compounds were present in the quantities shown in Table 1. Bioassays conducted with pure standards prepared in the laboratory, or purchased from commercial sources, indicated that these chemicals were indeed toxic to *N. ?dalbergiae* termites (Figure 9).

The toxic components isolated from *Dipterocarpus* resins have been found previously in other plant species (Shrimpton, 1973; Cook and Hain, 1988; Howard et al., 1988; Sukari and Takahashi, 1988; Farag et al., 1989; Komai and Tang, 1989). Laboratory bioassays conducted to assess the toxicity of these compounds with respect to attine ants and *Heliothis virescens* larvae have confirmed the toxicity of caryophyllene and caryophyllene oxide. Caryophyllene and caryophyllene oxide are growth inhibitors of *Heliothis virescens* (Gunasena et al., 1988). In contrast to the results reported here, bioassays with attine ants

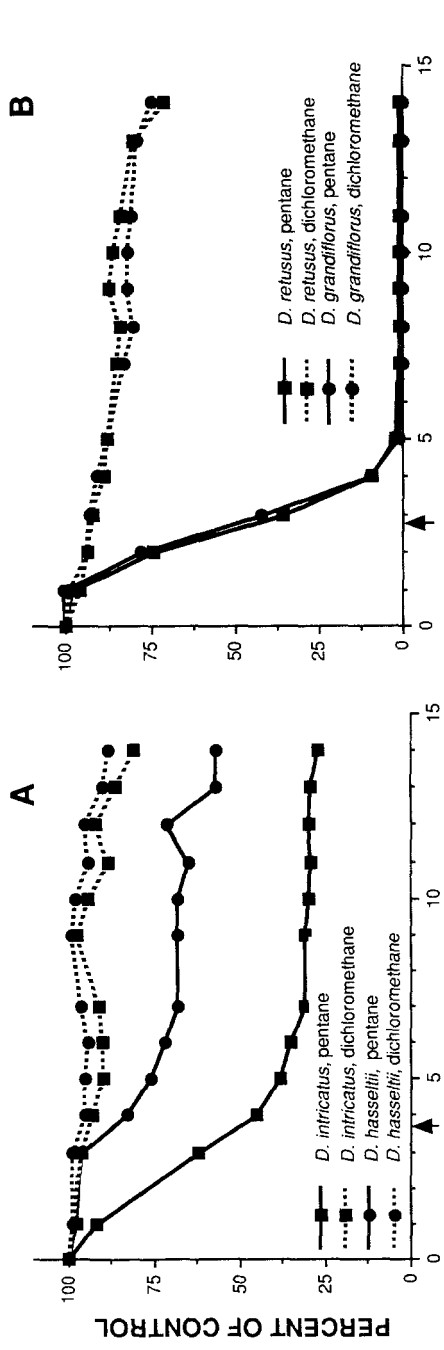


FIG. 7. Toxicity of *n*-pentane and dichloromethane fractions of *Dipterocarpus* spp. resins to *Neotermes ?dalbergiae* termites. Arrowheads on abscissa indicate the time point at which 50% of bioassay termites have died. (A) Fractions of *D. intricatus* and *D. hasseltii*. Arrowhead on the the abscissa indicates 50% point for *D. intricatus*; fractions of *D. hasseltii* did not cause this much death. (B) Fractions of *D. retusus* and *D. grandiflorus*; for the pentane fractions the 50% points are virtually identical.

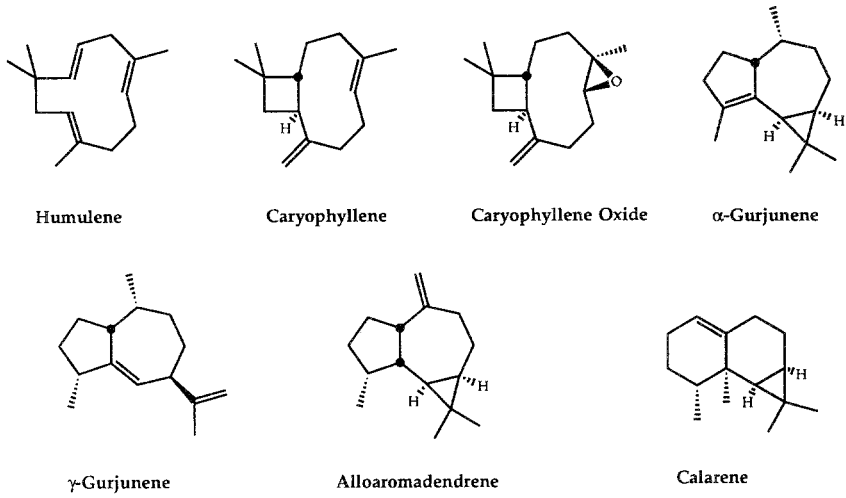


FIG. 8. Structure of chemicals isolated from resins of *Dipterocarpus* spp.

have shown that caryophyllene oxide was the more toxic compound (Howard et al., 1988). This contrasting result may be due to the fact that caryophyllene oxide is 20 times more repellent than caryophyllene in the ant case (Hubbell et al., 1983), and similar repellent effects could have reduced the amount of toxic chemical ingested by the *N. ?dalbergiae* termites. While caryophyllene oxide has been detected in dipterocarps (Bisset et al., 1966), in our study we found it only as a minor component of *D. retusus* (Table 1). Caryophyllene and other sesquiterpenes in the leaves of a Brazilian tree (*Copaifera langsdorfii*) have been demonstrated very recently to be significant elements of plant defense against herbivores (Macedo and Langenheim, 1989a,b).

TABLE 1. COMPONENTS (%) OF NONPOLAR FRACTIONS OF *Dipterocarpus* RESINS DETERMINED WITH CAPILLARY GC

	Dipterocarpus			
	retusus	intricatus	hasseltii	grandiflorus
Humulene	42	0	2	1
Caryophyllene	40	0	17	0
Caryophyllene oxide	1	0	0	0
α-Gurjunene	2	80	36	0
γ-Gurjunene	0	11	2	0
Alloaromadendrene	0	0	0	87
Calarene	2	0	35	1

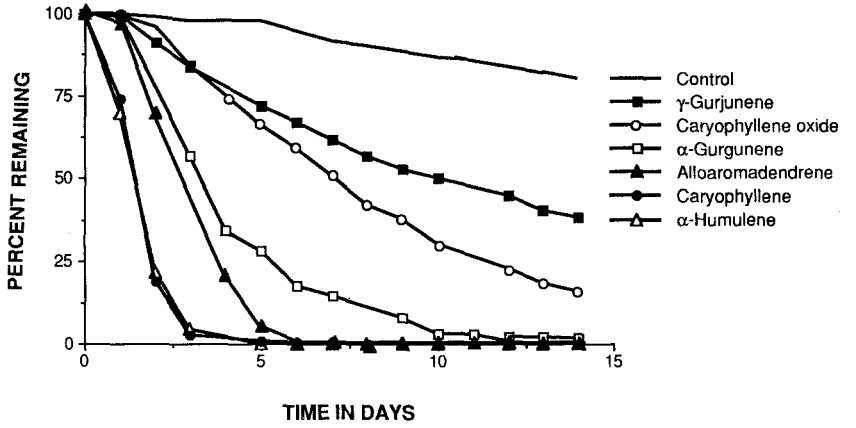


FIG. 9. Toxicity of authentic chemical standards to *Neotermes ?dalbergiae* termites.

Gross Effects of Toxic Resins

When fed to termites, biologically active resins, fractions, or pure resin components elicited three behavioral–physiological responses: copious passage of wet feces, sluggishness, and change in abdominal morphology. Within 24 hr of exposure to active compounds, termites had spotted copiously the filter papers with wet, dark brown feces. This was in contrast to termites in control dishes, which produced compact, dry, gray fecal pellets throughout the experiments. While termites in control groups moved actively in a dish when it was uncovered for counting, termites in treatment groups did not. This observation suggests that termites receiving chemical treatments responded in a general, nonspecific manner to resin chemicals. Specific physiological responses to applied chemicals might be expected to result in more accelerated effects, such as knockdown. Chemical treatments distorted the shape of the abdomen, changing it from biconvex, viewed laterally, to concave with respect to the ventral surface. The altered morphology of the abdomen appeared to result from the loss of abdominal contents.

Resin-Induced Hindgut Defaunation and Termite Death

Dissections of termites that had fed on filter papers treated with the biologically active *Dipterocarpus* resins showed that these insects lost their symbiotic protozoa as a result of the chemical treatments (Figures 10 and 11). It was desirable, then, to determine if the observed toxicity was due to the direct action of toxic chemicals on the termites or to the loss of protozoa. Loss of the symbiotic protozoa would result in death of the termite by starvation, as termites

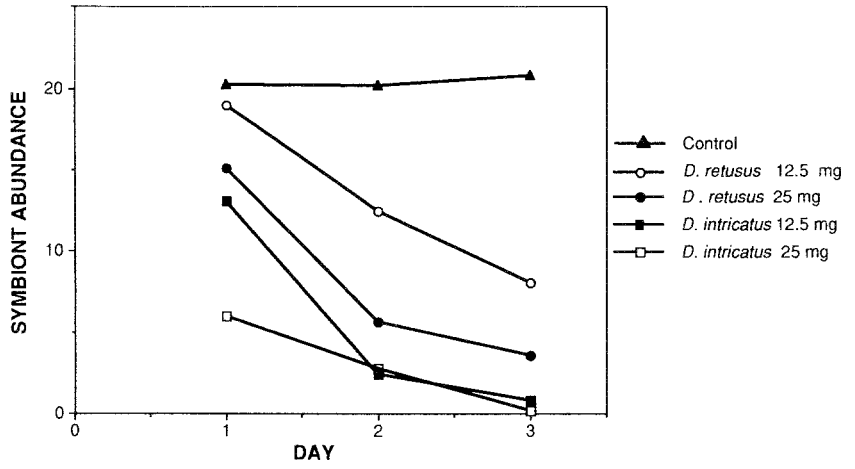


FIG. 10. Effect of crude resins of *Dipterocarpus* spp. on populations of symbiotic protozoa in the hindgut of *Neotermes ?dalbergiae*.

depend on these protozoa for the digestion of cellulose (Honigberg, 1970; Eutick et al., 1978).

Hyperbaric oxygen treatments, which rapidly remove the protozoa from the normally anaerobic hindgut paunch without killing the host termite, established that resin chemicals were probably acting directly on the insects. If removal of the protozoa were the cause of termite death, it would be expected

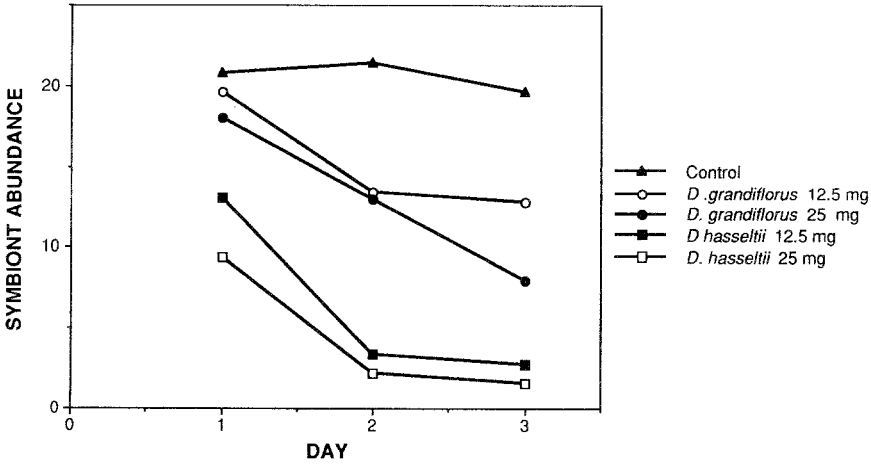


FIG. 11

that the oxygen-treated termites would die as rapidly as those that had fed on toxic components of tree resins. As shown in Figure 12, termites subjected to hyperbaric oxygen treatments died faster than controls (one-way ANOVA, $P < 0.0018$). However, complete defaunation of termites results in 50% of termite death after about 13 days (Figure 12), whereas toxic fractions may kill 50% of test termites in as little as three days (Figure 7B).

Comparison of Figure 12 to graphs of active fractions (Figure 7) shows that the termites that had been fed resin chemicals died at the same rate, or more rapidly, than those which had been defaunated. Nonpolar fractions of *D. hasseltii* (Figure 7A) were not statistically significantly more toxic to termites than hyperbaric oxygen treatments (one-way ANOVA, $P < 0.8861$). Fractions of *D. intricatus*, *D. retusus*, and *D. grandiflorus*, however, were more toxic to termites than the hyperbaric oxygen treatments ($P < 0.0039$). On the basis of this evidence it is possible to conclude that the toxic substances are acting primarily on the termite itself. Removal of the symbiotic protozoa may contribute to termite death, but it is not the main factor. While the chemicals do kill the symbiotic hindgut protozoa, the defaunation experiments indicate that removal of the protozoa alone is not sufficient to cause termite death within the short time frame seen in treatments with *D. intricatus*, *D. retusus*, and *D. grandiflorus*.

The apparent similarity in toxicity between nonpolar fractions of *D. hasseltii* (Figure 7) and hyperbaric oxygen treatments may result from the relatively

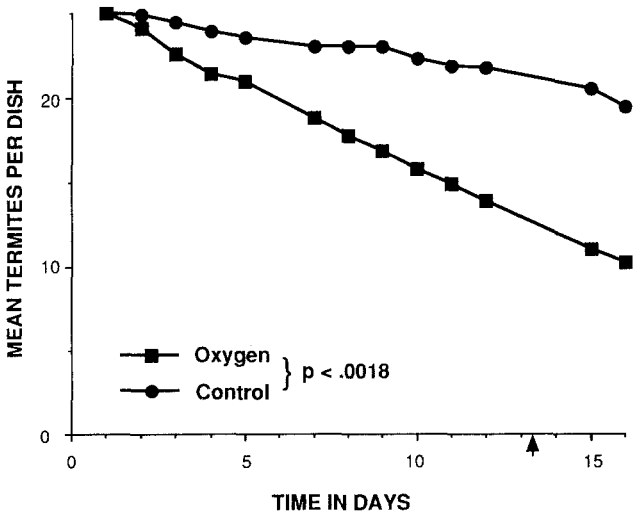


FIG. 12. Effect of hyperbaric oxygen treatment (100% O_2 at 300 kPA for 75 min) on survival of *Neotermes ?dalbergiae*. Control termites were not treated in any manner.

low concentrations of toxic chemicals present in the crude resins (Table 1). It is possible that while these chemicals are sufficiently toxic to remove protozoa (Figures 10 and 11), their concentrations are not high enough to cause much termite death.

Effects of Insecticide Synergists

Attempts were made to clarify the mode of action of these sesquiterpenes by feeding termites mixtures of 25 mg of crude resins with 10 mg of the insecticide synergist piperonyl butoxide (PBO). Extensive bioassays conducted with crude resins and purified fractions did not produce any evidence suggestive of synergism or antagonism (data not shown).

Modes of Action

Precise information on what the sesquiterpenes in this study do to metabolic processes is not available. Early studies of a number of terpene enones demonstrated a reciprocal relationship between solubility in water and the minimum concentration of a compound required to cause hemolysis (Dyson, 1930). Membrane damage, and subsequent disruption of aerobic bacterial energy transfer processes, results from treatment of *Rhodopsuedomonas sphaeroides* preparations with a variety of terpenoids (Knobloch et al., 1986). Dissolution of terpenoids in the phospholipid membranes of chloroplasts and mitochondria is said to be possible because cell walls do not form a barrier to the terpenoids. The terpenoids act variously as inhibitors of respiratory electron flow and uncouplers of oxidative phosphorylation (Knobloch et al., 1986).

Disruption of membrane-mediated processes might explain the mode of action of the chemicals with respect to both the *N. ?dalbergiae* termites and their symbiotic protozoa in the experiments reported here. Damage to protozoal membranes would result in death of the gut symbionts. Interference with membrane systems critical for nutrient and fluid transport (Dow, 1986; Phillips et al., 1986) and for regulation of ionic balance could cause symptoms consistent with those observed in the bioassay experiments reported here.

There is also the possibility that biotransformation might increase the toxicity of chemicals in dipterocarp resins. Fungal biotransformation of two of the main components of many dipterocarp resins, humulene and caryophyllene, has been reported (Abraham et al., 1985; Abraham and Stumpf, 1987; Sun et al., 1987). Caryophyllene did not display any biological activity, while its derivatives, caryophyllene alcohol and caryophyllone, caused potent and long-lasting antiasthmatic effects in guinea pigs in vivo and in vitro. It is possible that in the complex microbial environment of the termite hindgut, biotransformation reactions resulted in activation of chemicals that might otherwise be relatively benign.

Dosage of Toxic Compounds

It was not possible to estimate reliably the effective dose of each toxic chemical. Experiments designed to quantitate the amount of filter paper consumed by termites receiving chemical treatments failed. Termites in treatment groups deposited fecal slurry on filter papers. This made it impossible to determine how much of each paper was consumed, so that ingestion rates and estimated effective doses could not be calculated.

Taxonomic Distribution of Defensive Compounds

To what extent can the results reported here be generalized to other species in the Dipterocarpaceae, extending the argument that resins serve a defensive function? Given that all of the approximately 80 *Dipterocarpus* species so far examined (Bisset et al., 1966) contain at least one of the active compounds we identified (alloaromadendrene, caryophyllene, or humulene), the hypothesis that resins serve a defensive function seems to apply to that genus. Further, amounts of one or more of the same defensive compounds have been quantified in the genera *Anisoptera*, *Cotylelobium*, *Dryobalanops*, *Shorea*, and *Upuna* (Bisset et al., 1967).

With the exception of the African genera *Marquesia* and *Monotes*, all dipterocarp woods contain resin canals (Bancroft, 1935; Metcalfe and Chalk, 1979). These axial, intercellular canals, encountered in few other groups, are a regular, not a traumatic, feature, and transport resin through the petiole to the lamina of the leaf (Brazier, 1979; Metcalfe and Chalk, 1979). As such, the resin canals may form part of a constituitive defense system directed against biological attack in general. An on-going system of defense against biological attack, based on the circulation of protective chemicals through the wood, might be expected to be active from an early age. This conjecture is supported by developmental information–embryonic dipterocarps of resin-producing genera possess resin canals (Solereder, 1908; Maury-Lechon, 1979). Thus, resin-producing dipterocarps meet this criterion.

Continuous production of defensive terpenes throughout the tree, as part of a baseline level of chemical protection, requires appropriate structures for the distribution of these chemicals. As in the case of the maritime pine, *Pinus pinaster* Ait. (Cheniclet, 1987), synthesis of resins in dipterocarps is likely not localized, so resin canals are necessary to transport these materials. The canals also serve to keep the toxic resins from contacting sensitive plant tissues (Knobloch et al., 1986).

In addition to the constituitive defenses offered by the baseline levels of circulating sesquiterpenes, wounding may result in formation of traumatic radial canals needed to transport newly synthesized resin to a wound site (Brazier, 1979). Increased resin exudation, in response to biotic and artificial elicitors,

has been demonstrated for the dipterocarp *S. javanica*, and other species (Messer, 1990).

If sesquiterpenes of the Dipterocarpaceae fulfill a defensive role, do they fit into the conceptual framework Feeny (1976) established for plant defensive compounds? They appear to. Resin composition varies broadly over both geographic and taxonomic ranges (Bisset et al., 1966, 1967, 1971; Diaz et al., 1966). Thus the expectation of chemical diversity in the face of herbivores is met. What data exist also indicate that dipterocarp resins represent plant defenses of the quantitative type (Feeny, 1976).

The research reported here considered only nonpolar sesquiterpenes of dipterocarp resins, which represent only a portion of the chemicals present in the resin. We demonstrated that some of the chemicals shown in Figure 8 have biological activity against termites and thus may function in a defensive role, possibly in concert with compounds contained in the bark (Geewananda et al., 1986, Bokel et al., 1988). Because resins also are found in seeds, they may contribute to chemical defense against seed predation as well.

While the role of more polar constituents, for example, triterpene alcohols, is not known, evidence from other systems suggest that these compounds also may contribute to defense (Baas, 1985; Kapoor and Chawla, 1986). In other contexts the chemicals also show biological activity: dipterocarp resin triterpenoids inhibit herpes virus growth (Poehland et al., 1987).

Finally, triterpenoid chemicals may modulate competition between tree species through indirect means. Found in many Dipterocarpaceae, the triterpene alcohol dipterocarpol (Bandaranyake et al., 1975) induces growth in culture of the microsymbiont *Frankia* from actinorhizal root nodules (Quispel et al., 1989). Mycorrhizal associations have been suggested as a factor underlying the persistent dominance of a few tree species in tropical primary forests; dipterocarps are an excellent example of this phenomenon (Connell and Lowman, 1989). Thus it is tempting to speculate that the production of dipterocarpol has enabled dipterocarps to establish and maintain superior mycorrhizal associations, with the result that these trees often dominate lowland forests in Southeast Asia.

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RUST-RED GRAIN BEETLE, *Cryptolestes ferrugineus*, AND FLAT GRAIN BEETLE, *Cryptolestes pusillus*: ANTENNAL AND BEHAVIORAL RESPONSES TO SYNTHETIC COMPONENTS OF THEIR AGGREGATION PHEROMONES

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Abstract—Antennal and behavioral responses of the rust-red grain beetle, *Cryptolestes ferrugineus*, and the flat grain beetle, *C. pusillus*, to synthetic samples of the macrocyclic lactones reported to comprise their aggregation pheromones were investigated. Electroantennogram (EAG) recordings were obtained successfully from both species for the first time. Females of both species showed larger EAGs than males. The EAGs of *C. ferrugineus* showed a high degree of specificity for conspecific aggregation pheromone components; *C. pusillus* showed much less specificity. Behavioral tests were conducted using two-choice pitfall bioassays. Separation of the results into the two effects of activity stimulation and direction finding showed that both effects contributed to the overall response, although sometimes to different extents. The strain of *C. pusillus* studied responded equally well to both components of its pheromone, whereas it had been reported previously that only one was active, the other acting as a synergist and eliciting no response when tested alone. With both species, behavioral response was elicited with a single lactone, suggesting that it might not be necessary to use both components for field use. Particularly surprising was that *C. pusillus* showed a greater

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response to the pheromone components of *C. ferrugineus* than to its own. Aeration of the two species and thermal desorption of the collected volatiles confirmed production of the expected lactones, and aeration of authentic lactones showed that the response was not due to the *C. ferrugineus* pheromone components being markedly more volatile. This response, which seems to be an actual preference, is the first to be discovered among the cucujid beetles and encourages optimism that a practical lure for various species may not need to be as complex as originally feared.

Key Words—*Cryptolestes ferrugineus*, *C. pusillus*, Coleoptera, Cucujidae, aggregation pheromone, macrocyclic lactone, electroantennogram, behavioural bioassay, interspecific response, thermal desorption.

INTRODUCTION

The rust-red grain beetle, *Cryptolestes ferrugineus* (Stephens), and the flat grain beetle, *Cryptolestes pusillus* (Schonherr), are serious pests of stored grain and grain products (Sinha and Watters, 1985). In response to pressure from the cereal trade (Cogan and Chambers, 1988), it is hoped to improve the detection of such pests by enhancing trap effectiveness with attractive chemicals such as pheromones. To ensure the best possible enhancement, it is first necessary to obtain detailed knowledge of the response to these chemicals in laboratory tests.

The production of an aggregation pheromone by adult male *C. ferrugineus* was first reported by Borden et al. (1979), and it has since been identified as a mixture of two synergistic macrocyclic lactones, 3(*Z*),11(*S*)-dodecen-11-olide (I) and 4(*E*),8(*E*)-4,8-dimethyldecadienolide (V) [Wong et al., 1983; Oehlschlager et al., 1983, 1987; Roman numbering according to Pierce et al. (1984)]. The response of mixed-sex adult *C. ferrugineus* to these lactones has been assessed in the laboratory with a two-choice pitfall bioassay. Individually I and V each elicited an attractive response, but there was a higher response when they were tested together as a 1 : 1 mixture (Wong et al., 1983). In a larger test, racemic I and V in the ratio of 1 : 1.6 [(*S*)-I : V] emitted from a hollow fiber at a rate of approximately 30 male equivalents in a newly designed enclosed chamber trap were effective in recapturing *C. ferrugineus* released in floor areas of 2.5 × 1.9 m (Lindgren et al., 1985). Finally, mixtures of racemic I and V in ratios of 1 : 18 [(*S*)-I : V] in insect detection traps (Loschiavo and Atkinson, 1973) at a depth of 30–60 cm in grain in primary elevator annex bins caught significantly more *C. ferrugineus* than unbaited traps (Loschiavo et al., 1986).

The pheromone of *C. pusillus* bears a strong resemblance to that of *C. ferrugineus*. Millar et al. (1985) reported that adult male *C. pusillus* produce an aggregation pheromone, the components being the two macrocyclic lactones, 3(*Z*)-dodecenolide (VI) and 5(*Z*),13(*S*)-tetradecen-13-olide (VII) (Millar et al., 1983; Oehlschlager et al., 1987). In laboratory tests, lactone VI was found to

be attractive alone, and lactone VII synergized the response to VI but was not active alone. In contrast, we have found that a synthetic sample of lactone VII was quite attractive to *C. pusillus*, and preliminary details have already been reported (Pinniger and Chambers, 1987). This emphasizes the need for detailed understanding of these pheromone systems.

In an earlier investigation of the potential of stored-product beetle pheromones for practical use, we studied the saw-toothed grain beetle, *Oryzaephilus surinamensis* (L.), whose aggregation pheromone includes a mixture of 3(*Z*),6(*Z*),11(*R*)-dodecadien-11-olide (II), 3(*Z*),6(*Z*)-dodecadienolide (III), and 5(*Z*),8(*Z*),13(*R*)-tetradecadien-13-olide (IV) (Oehlschlager et al., 1987). We found that the proportions of the components differed from those published previously and that electroantennogram (EAG) techniques could be used to investigate this system (White et al., 1989). Detailed study has begun to reveal the role of the individual components (White and Chambers, 1989). Despite the convenient label of aggregation pheromone, it seems that the mixture acts in part as a sex pheromone. This would appear not to be the case for *C. pusillus* where, surprisingly, the male-produced pheromone was thought to elicit a greater response from males than females (Millar et al., 1985).

The purposes of the present study were to continue our detailed investigation of responses to stored product beetle pheromones in the laboratory by establishing whether it would be possible to study *C. ferrugineus* and *C. pusillus* by EAG techniques and by investigating whether there were any further differences with previously published work in the behavioral responses of these two species to synthetic components of their pheromones.

METHODS AND MATERIALS

Insects. *Cryptolestes ferrugineus* and *C. pusillus* were maintained in mixed-sex culture at 25°C. Both species were reared on a 5:5:1 mixture of rolled oats, wheatfeed, and brewers' yeast at an average density of 3000 adults/kg food. For EAG recordings, insects were used when 4–8 weeks after adult eclosion. The sex of individuals was determined by the mandibular tooth present in males of *C. ferrugineus* and the longer antennae of males in *C. pusillus* (Halstead, 1963). For the behavioral bioassays, *C. ferrugineus* were used when 0–7 weeks and the *C. pusillus* when 2–4 weeks after adult eclosion. Insects were removed from culture, put into glass tubes, and deprived of food for the 24 hr before testing.

Chemicals. All the macrocyclic lactones are referred to by the Roman numeral notation of Pierce et al. (1984). Samples of the *C. ferrugineus* lactones I [3(*Z*),11(*S*)-dodecen-11-olide] and V [4(*E*),8(*E*)-4,8-dimethyl-decadienolide] were synthesized by Sakai and Mori (1986). The *C. pusillus* lactones VI

[3(*Z*)-dodecenolide] and VII [5(*Z*),13(*S*)-tetradecen-13-olide] were synthesized by Sakai et al. (1986). Lactones II [3(*Z*),6(*Z*),11(*RS*)-dodecadien-11-olide], III [3(*Z*),6(*Z*)-dodecadienolide], and IV [5(*Z*),8(*Z*),13(*RS*)-tetradecadien-13-olide] were synthesized by Millar and Oehlschlager (1984) and supplied by Dr. A.M. Pierce (Simon Fraser University, Burnaby, British Columbia, Canada V5A 1S6).

Electroantennogram (EAG) Procedure. The EAG system used was essentially similar to that previously described (White and Birch, 1987; White et al., 1989). The indifferent electrode was inserted through the soft intersegmental cuticle between the head and thorax. Test material was applied in 10 μl of pentane (Gold Label Spectrophotometric Grade, Aldrich Chemical Co. Ltd), allowing 30 sec for the solvent to evaporate before the paper cartridge was placed in the delivery pipette. Blank cartridges were made up using 10 μl of solvent only. All cartridges were made up fresh for every test and used once only.

EAG Experiments. Each insect preparation was used for two separate EAG experiments. Experiment 1 investigated the EAGs of both species to a range of concentrations of the identified aggregation pheromone components of *C. ferrugineus* and *C. pusillus*, i.e., lactones I, V, VI, and VII. Each lactone was presented at 6 log doses in ascending order from 0.01 ng to 1000 ng. The order of presentation of the lactones was changed randomly between preparations. Prior to each test stimulus, a blank cartridge (solvent only) was presented, and the EAG recorded was subtracted from the subsequent test response to allow for the response to the solvent and the mechanical disruption of the air flow.

In experiment 2 each preparation was presented with a 1000-ng dose of each of the seven macrocyclic lactones I–VII. As for the previous experiment, the order of presentation was changed randomly between preparations and the blank response was subtracted before the results were analyzed.

Both experiments were conducted on a total of 20 preparations, using five individuals of each sex, for each of the two species, in random order. Results from both experiments were subjected to a split-plot factorial analysis of variance (Sokal and Rohlf, 1969) to identify the factors that affected the EAG response.

Pitfall Bioassay Procedure. Behavioral response to the lactones was assessed using a two-choice pitfall bioassay similar to that described by White and Birch (1987). Filter paper disks of 1.5 cm diameter were treated with each test stimulus (lactone or lactone mix) in 5 μl purified pentane or with 5 μl purified pentane alone (blank disks). After allowing 1–3 min for the solvent to evaporate, the disks were placed at the bottom of the pitfall tubes.

Twenty insects of mixed sex were introduced into each arena, and the apparatus was immediately covered by a 10-cm-diameter Petri dish. All tests were carried out at 25°C and 70% relative humidity under low-intensity red

light. After 1 hr the numbers of insects left in the arena (nonresponders) and in each tube (responders) were counted. Except where otherwise stated, 15 replicates were carried out for each test. The tests were run in the afternoon and the insects were used once only, then discarded.

Pitfall Bioassay Experiments. Four experiments were undertaken using the two-choice pitfall bioassay method. In experiment 3, *C. ferrugineus* was tested with blank disks in one pitfall and the *C. ferrugineus* lactones in the other. In experiment 4, *C. pusillus* was tested using blank disks in one pitfall and the *C. pusillus* lactones in the other. In Experiment 5, *C. ferrugineus* and *C. pusillus* were tested separately with a mixture of the *C. ferrugineus* lactones in one pitfall and a mixture of the *C. pusillus* lactones in the other. Lastly, in experiment 6, *C. pusillus* was tested using blank disks on one pitfall and either a mixture of the *C. ferrugineus* or the *C. pusillus* lactones in the other.

The results from the pitfall bioassay experiments were subjected to binomial logistic analysis, allowing for over- or under-dispersion of the data where necessary, using the Generalised Linear Interactive Modelling package of the Royal Statistical Society of London. A first analysis was undertaken as a measure of activity by comparing the numbers of nonresponders and responders (insects that were found in either pitfall). A second analysis was used to assess the directional nature of the response by comparing the numbers of insects in the two pitfall tubes taken separately. Where necessary, probability values were assigned to comparisons of means by using the z test before back transformation.

Aeration Experiments. In the first aeration experiment the volatilities of lactones I, V, VI, and VII were compared. Solutions of about 10 ng/μl of the lactones in pentane were analyzed by splitless injection on a Varian 3700 gas chromatograph and the concentrations adjusted until all the lactones gave approximately the same mean peak area. Pieces of filter paper (40 $\times$ 3 mm) with 2 μl of these lactone solutions and about 25 ng of methyl laurate as internal standard were placed in a glass tube (50 $\times$ 3.5 mm ID) and aerated for 20 min in an all-glass apparatus with purified air at 0.5 liters/min. Volatiles were collected in borosilicate glass tubes (220 $\times$ 3.5 mm ID) containing 150 mg preconditioned 60/80 mesh Tenax-TA (Chrompack UK Ltd). Preliminary experiments with two of these tubes in tandem confirmed that a single tube was sufficient to absorb all the volatiles in these experiments. Trapped volatiles were desorbed by heating at 220°C for 3 min in a stream of about 8 ml/min nitrogen. Desorbed volatiles were flushed into a Varian 3700 gas chromatograph, cold-focused, then analyzed. Lactones were analyzed in the order I, VI, V, and VII to allow any carryover from one injection to the next to be readily detected, and this sequence was repeated twice.

In the second aeration experiment, batches of 200 adult male *C. ferrugineus* or *C. pusillus* were placed in a glass aeration chamber (105 $\times$ 25 mm ID)

with about 0.1 g of the standard culture food. After allowing to settle for 10 min they were aerated at an average temperature of 20°C with air at about 0.3 liters/min in apparatus similar to that used above except that the incoming air was humidified. Aeration was continued for either 1 hr (*C. ferrugineus*) or 2 hr (*C. pusillus*). Volatiles were collected, thermally desorbed, and analyzed as above. Insects were aerated only once, then discarded. There were three replicate aerations for each species, examined alternately, and all the aerations were started at about the same time of day. The amounts of lactones produced were calculated by comparison of mean peak area with that obtained from direct injections of a standard solution of methyl laurate.

RESULTS

EAG Response to Macrolide Lactones. The EAGs of both sexes of *C. pusillus* and *C. ferrugineus* to lactones I, V, VI, and VII (experiment 1) are shown in Figure 1. There was a significant difference between the responses of males and females in both species ($F = 7.1$, $P < 0.05$) with females producing EAGs of greater amplitude. Within each species, however, the overall patterns of the dose–response curves were similar.

There were marked differences between the responses of the two species, however, shown in the analysis of variance by a highly significant interaction between the species of the responder and the different lactones tested ($F = 102.2$, 3 *df*, $P < 0.001$). Examination of Figure 1A and B shows that lactone VI elicited one of the largest EAG responses in *C. pusillus*. At low doses, lactone VII was second in EAG activity, but at doses above 1 ng, lactone V caused a larger EAG, approaching that of lactone VI.

The EAGs of *C. ferrugineus* were markedly different (Figure 1C and D), with lactones I and V showing the lowest response thresholds and eliciting the largest EAGs. In contrast, lactones VI and VII produced small EAGs even at the highest doses tested.

This difference in response specificity was also observed in experiment 2, which investigated the EAGs of both species to a single dose of seven different lactones (Figure 2). As before, the EAGs of females were significantly greater than those of males in both species ($F = 9.1$, $P < 0.01$) (Table 1, Figure 2). There was also a highly significant interaction between the species tested and the lactones presented ($F = 15.6$, $P < 0.001$, Table 1), demonstrating the difference in the response spectra for the two *Cryptolestes* species. Examination of Figure 2 confirms the findings of the first experiment in that *C. pusillus* responds more strongly at the EAG level to lactones V and VI, but also responds to lactone VII and the other four lactones presented. *C. ferrugineus* responded

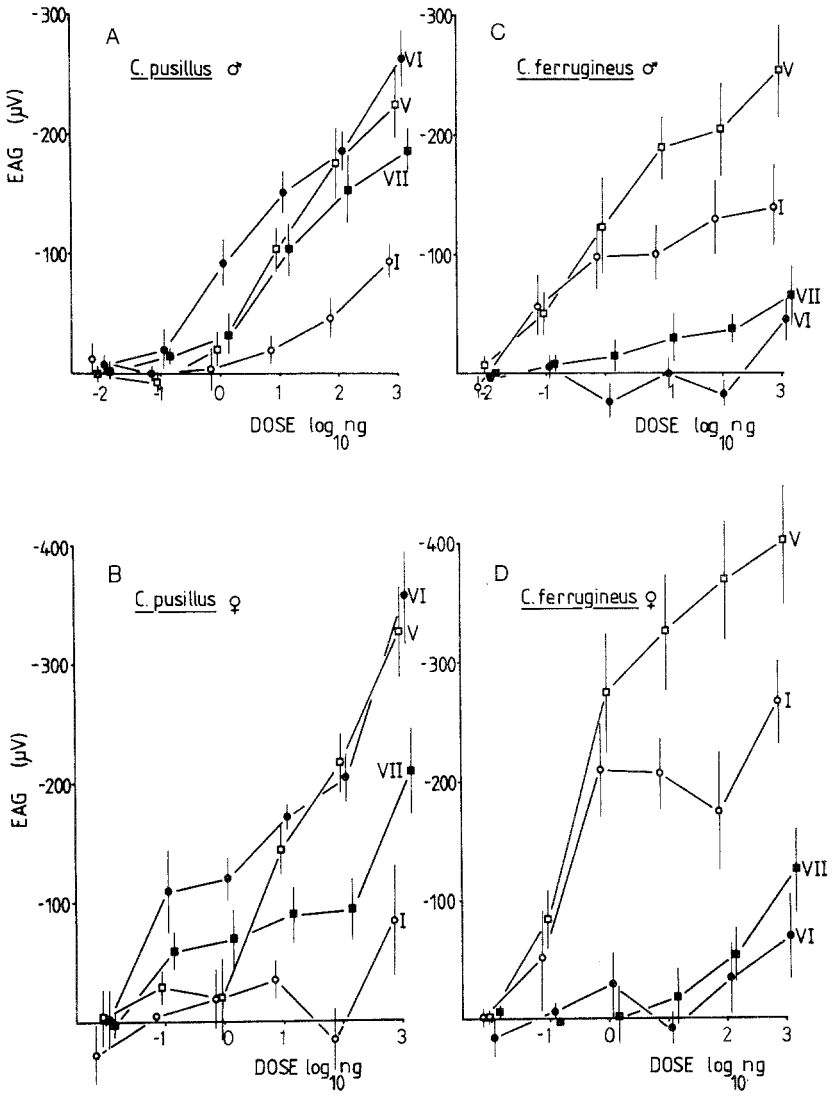


FIG. 1. A–D EAG response of *C. pusillus* and *C. ferrugineus* to a range of six doses of lactones I, V, VI, and VII (experiment 1). Each point gives mean ± SE for five individuals.

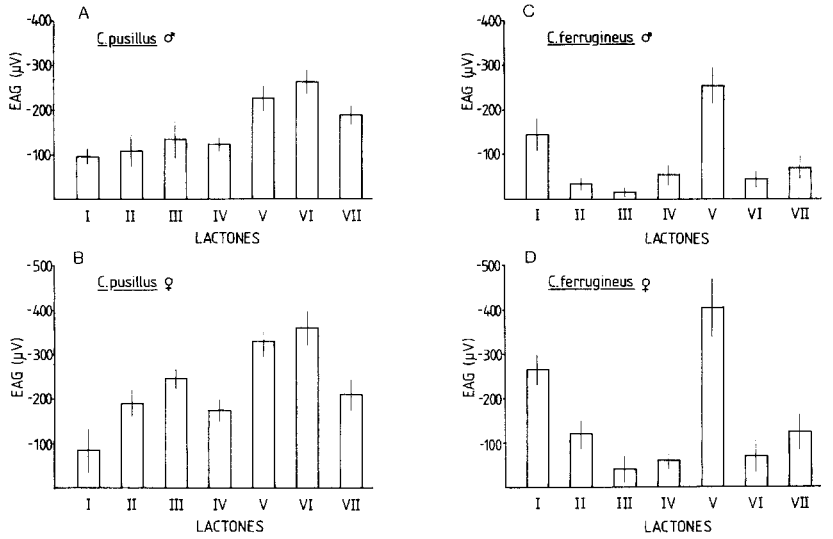


FIG. 2. A–D EAG response of *C. pusillus* and *C. ferrugineus* to a 1000 ng dose of lactones I–VII (experiment 2). Each bar gives mean ± SE for five individuals.

most strongly to lactone V followed by lactone I, with less response to the other lactones.

Pitfall Bioassay Responses. Within each experiment, when more tests were needed than could be run on one day, variability in results with the same source

TABLE 1. FACTORIAL SPLIT-PLOT ANOVA TABLE FOR EFFECT OF SPECIES AND SEX ON EAG RESPONSE TO LACTONES I–VII (EXPERIMENT 2)

Source	*df*	*F*
Species of responder	1	14.5[a]
Sex of responder	1	9.1[a]
Sex × species	1	0.0
Residual	16	
Individuals	19	
Lactones	6	26.5[b]
Species × lactones	6	15.6[b]
Sex × lactones	6	1.8
Species × sex × lactones	6	1.2
Residual	96	
Total	139	

[a] $P < 0.01$.
[b] $P < 0.001$.

between days was less than within days. Combination of results across days was therefore valid. Figure 3A shows the results of experiment 3 analyzed for activity (i.e., nonresponders vs. responders to either pitfall combined) expressed as mean percentage response with approximate 95% confidence intervals. Dif-

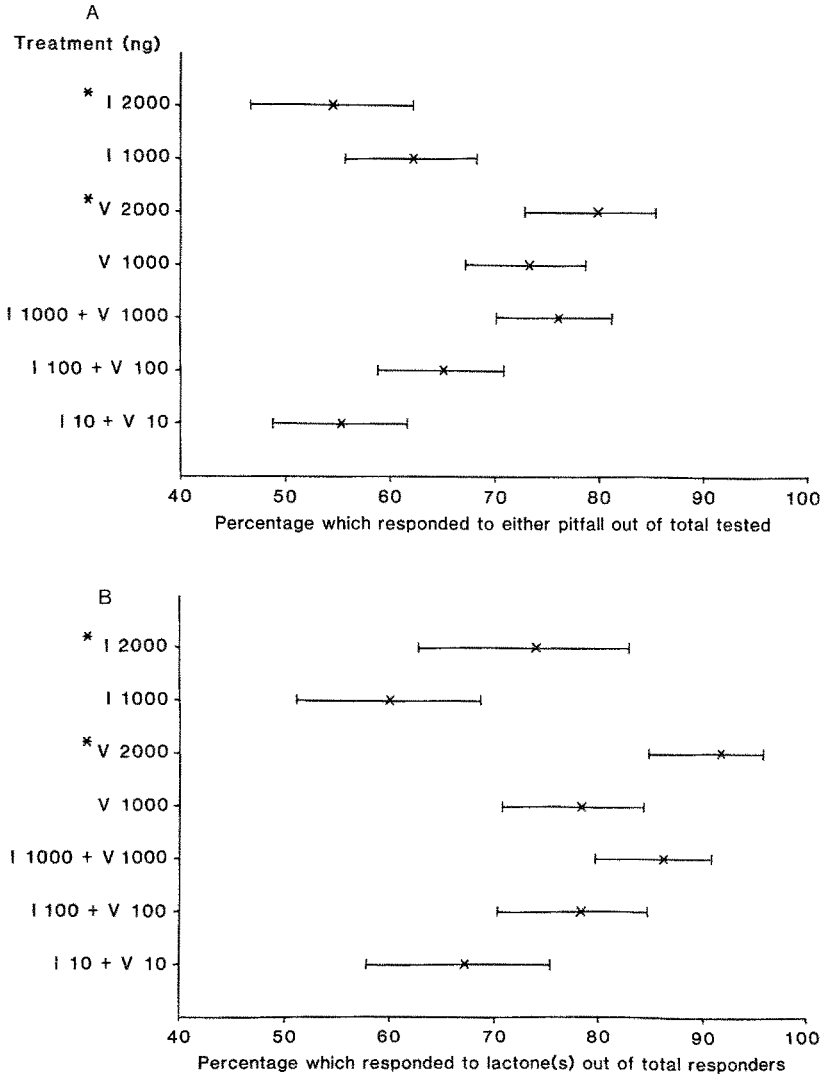


FIG. 3. Pitfall bioassay experiment 3, *C. ferrugineus*: binomial logistic analysis means with approximate 95% confidence intervals, $N = 15$ (*10), for (A) responders to either pitfall vs. nonresponders and (B) responders to lactones vs. responders to blank disks.

ferences between the various stimuli were significant ($\chi^2 = 66.6$, 6 *df*, $P < 0.005$). The activities when the lactones were tested separately at 1000 ng were similar to the activity in a separate experiment with blank disks in both pitfalls (mean percentage response 73.2%, approximate 95% confidence intervals 80.0 and 65.2%; $N = 15$). This separate experiment provided a means of assessing the normal activity level of the species under the same test conditions but without any attractant, arrestant, or repellent effect of the lactones. However, the activity with 1000 ng of lactone V was significantly greater than that with 1000 ng of lactone I ($z = 2.51$, $P < 0.01$). With the mixture of lactones I and V at 1000 ng of each, the activity was markedly higher than with the mixture at 10 ng of each ($z = 4.65$, $P < 6 \times 10^{-5}$), and it was significantly greater than the activity with lactone I at 1000 ng ($z = 3.20$, $P < 1.4 \times 10^{-3}$), but it was not significantly better than the activity with lactone V at 1000 ng. The activity with the individual lactones was not significantly better at 2000 ng than at 1000 ng.

The directional nature of the responses in experiment 3 is shown in Figure 3B, expressed as the percentage of responders found in the pitfall containing the lactone(s). Again the differences between the stimuli were significant ($\chi^2 = 70.6$, 6 *df*, $P < 0.005$). In the separate experiment with blank disks in both pitfalls, there was no evidence for the response having any directional nature (mean percentage response 48.4% to one of the pitfalls, approximate 95% confidence intervals 60.9 and 36.0%; $N = 15$). However, there was some evidence for directional response towards lactone I at 1000 ng (mean percentage response 60.2%) and stronger evidence in the case of lactone V at 1000 ng (mean percentage response 78.4%). The directional response to lactones I and V when mixed together at 1000 ng of each, was significantly stronger than that to the mixture at 10 ng of each ($z = 3.54$, $P < 4 \times 10^{-4}$) and that to lactone I alone at 1000 ng ($z = 4.68$, $P < 6 \times 10^{-5}$), but it was not significantly better than the directional response to lactone V alone at 1000 ng. Doubling the doses of the individual lactones to 2000 ng made no significant difference to the response towards lactone I, but there was a significant improvement towards lactone V ($z = 2.73$, $P < 6.3 \times 10^{-3}$). However, the response towards lactone V at 2000 ng was not significantly better than that towards the mixture of lactones I and V at 1000 ng of each.

Figure 4A shows the results of experiment 4 analyzed for activity. Differences between the stimuli were significant ($\chi^2 = 151.8$, 6 *df*, $P < 0.005$). The activities with the lactones tested separately were very similar to that found in a separate experiment with blank disks in both pitfalls (mean percentage response 84.4%, approximate 95% confidence intervals 90.0 and 76.5%; $N = 15$). The activity with lactone VI at 2000 ng was not significantly different from that with lactone VII at 100 ng. With the mixture of the two lactones in these amounts, the activity was not significantly different from that with lactone VI alone at

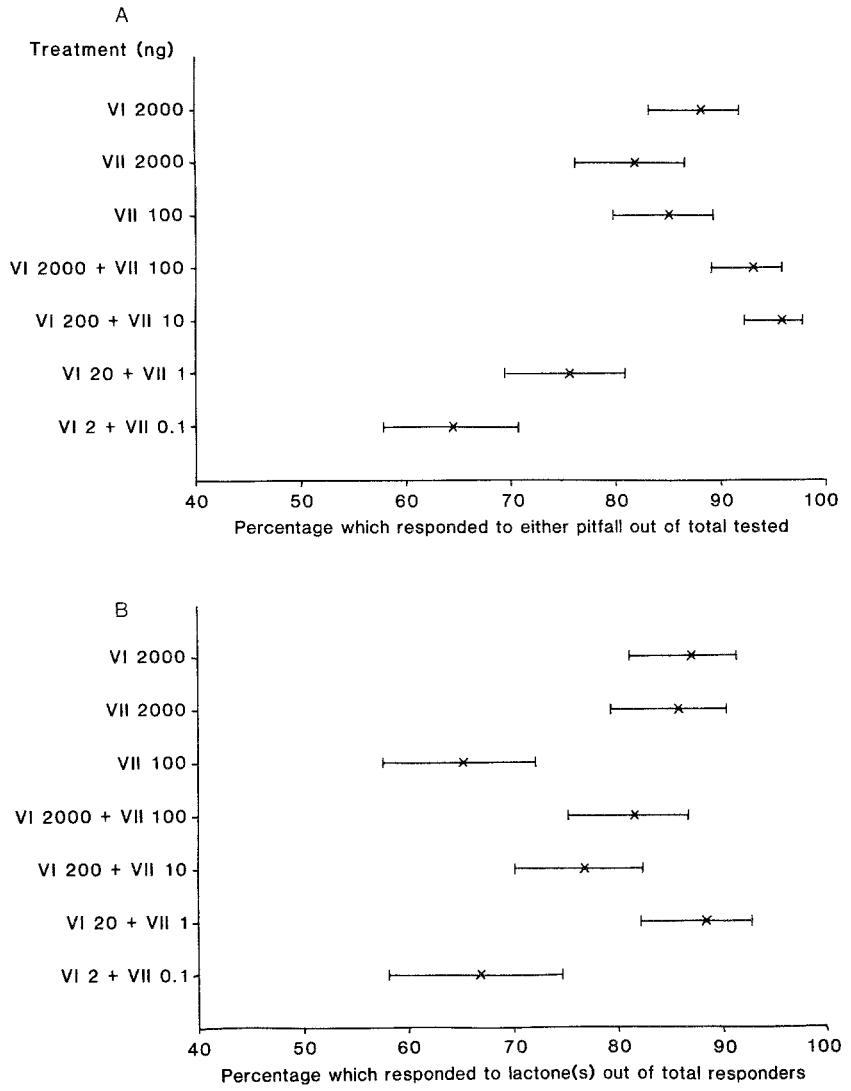


FIG. 4. Pitfall bioassay experiment 4, *C. pusillus*: binomial logistic analysis means with approximate 95% confidence intervals, $N = 15$, for (A) responders to either pitfall vs. nonresponders and (B) responders to lactones vs. responders to blank disks.

2000 ng, but it was significantly better than that with lactone VII alone at 100 ng ($z = 2.63$, $P < 8.5 \times 10^{-3}$). The activity was not altered significantly in going from the mixture of lactones VI and VII at 2000 and 100 ng, respectively, to that with 200 and 10 ng, respectively, but it did fall significantly in going

from there to the mixture with 2 and 0.1 ng respectively ($z = 6.83$, $P < 6 \times 10^{-5}$).

The directional nature of the responses in experiment 4 is shown in Figure 4B. Again the differences between the stimuli were significant ($\chi^2 = 75.4$, 6 *df*, $P < 0.005$). In the separate experiment with blank disks in both pitfalls, the mean percentage response to one of the pitfalls was 43.3%, with the approximate 95% confidence intervals of 51.0 and 35.9% ($N = 15$), confirming that there was no significant directional nature to the response. However, the directional response to lactone VII alone at 100 ng was noticeable, while that to lactone VI at 2000 ng was strong and the difference between these two was significant ($z = 4.50$, $P < 6 \times 10^{-5}$). While the directional response towards the mixture of 2000 ng of lactone VI and 100 ng of lactone VII was significantly better than that to 100 ng of lactone VII alone ($z = 3.36$, $P < 7.8 \times 10^{-4}$), it was not significantly better than that to 2000 ng of lactone VI alone. The directional response did not change significantly in going from the mixture of lactones VI and VII with 2000 and 100 ng, respectively, to that with 20 and 1 ng, respectively, but it did fall significantly in going from there to the mixture with just 2 and 0.1 ng, respectively ($z = 4.10$, $P < 6 \times 10^{-5}$). Interestingly, when lactone VII was tested at 2000 ng, it was found to be no different from lactone VI at the same dose.

In experiment 5, the activities of the two species (Figure 5A) were similar to those that had been observed in the separate tests with blank disks in both pitfalls. In the present experiment there was no significant difference in activity between the two species ($\chi^2 = 0.4$, 1 *df*). Figure 5B shows the results expressed in terms of the directional response towards lactones I and V. As expected, *C. ferrugineus* showed a marked preference for its own lactones (mean percentage response towards I and V, 69.8%). Most surprisingly, *C. pusillus* showed the same marked preference for the *C. ferrugineus* lactones over its own (mean percentage response towards I and V, 71.3%); in fact, there was no significant difference between the directional responses of the two species ($\chi^2 = 0.11$, 1 *df*).

In experiment 6, the activity of *C. pusillus* with lactones I and V was similar to that in the separate test with blank disks in both pitfalls. However, the activity in the test with lactones VI and VII was lower than expected and was in fact significantly less than that with lactones I and V ($z = 3.72$, $P < 2 \times 10^{-4}$) (Figure 6A). *C. pusillus* showed a strong directional response towards lactones VI and VII (Figure 6B), but the response towards the *C. ferrugineus* lactones I and V was even stronger, the difference being highly significant ($z = 4.16$, $P < 6 \times 10^{-5}$).

Aeration Experiments. The mean peak areas of the lactone solutions after analysis by direct injection and adjustment of concentrations were: I 190, V 175, VI 180, and VII 175 FID area counts $\times 10^{-3}/\mu$l. In each case the standard error of the mean was about 5%. This precaution ensured that any differences

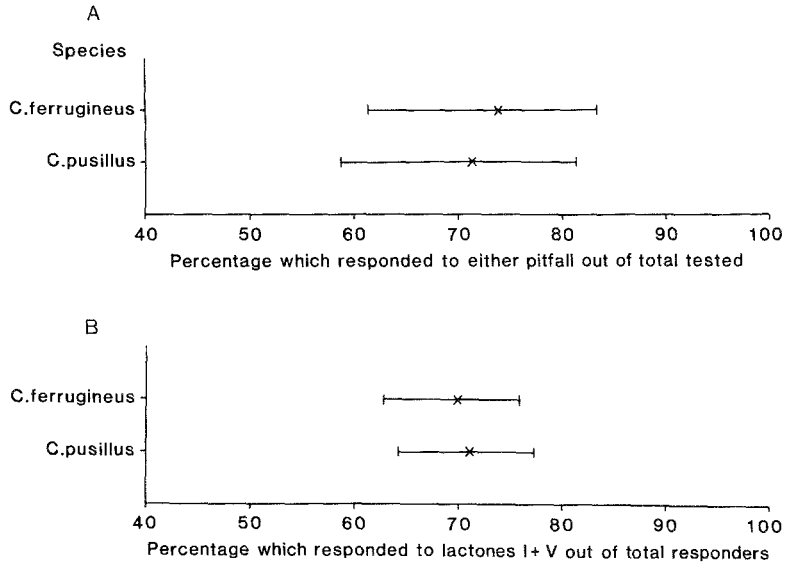


FIG. 5. Pitfall bioassay experiment 5, *C. ferrugineus* and *C. pusillus*: binomial logistic analysis means with approximate 95% confidence intervals, $N = 15$, for (A) responders to either pitfall vs. nonresponders and (B) responders to lactones I 100 + V 100 ng vs. responders to lactones VI 190.5 + VII 9.5 ng.

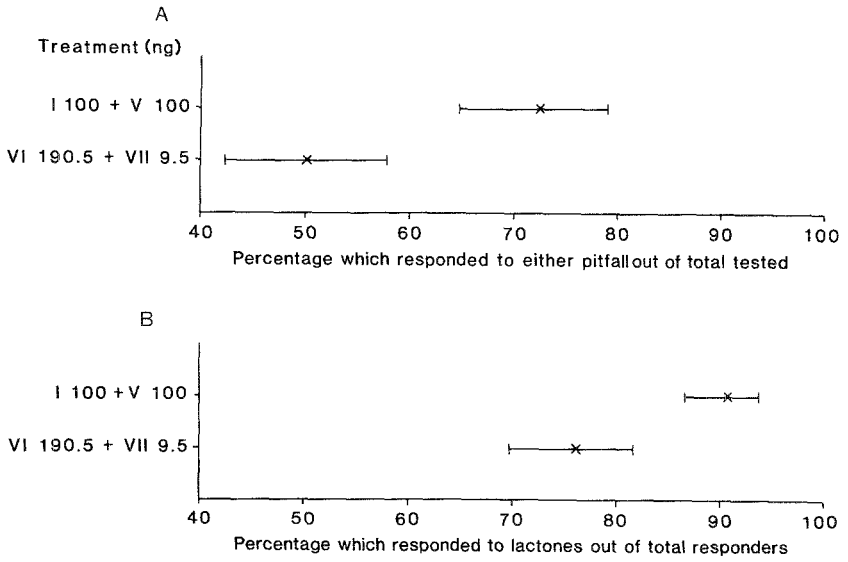


FIG. 6. Pitfall bioassay experiment 6, *C. pusillus*: binomial logistic analysis means with approximate 95% confidence intervals, $N = 15$, for (A) responders to either pitfall vs. nonresponders and (B) responders to lactones vs. responders to blank disks.

found subsequently would not be due to differences in FID response to the four lactones. Throughout the aeration experiments at least 99% of the removable volatiles were desorbed from each Tenax tube during the first desorption, and the quoted results therefore are based on these alone. To allow for variations in the performance of the thermal desorption apparatus, the mean peak areas of the lactone solutions after aeration were corrected for variations in the mean peak area of the methyl laurate internal standard. It was felt that any remaining differences between the lactones would be due solely to differences in volatility. The corrected mean peak areas from the aerated lactones were: I 130, V 140, VI 215, and VII 90 FID area counts $\times 10^{-3}/\mu$l.

Aeration of the insects on 0.1 g food gave large GC peaks whose retention times were similar to those of the authentic lactones. Mean retention times were: *C. ferrugineus* 14.29 min (lactone I 14.23 min) and 14.38 min (lactone V 14.32 min); *C. pusillus* 15.11 min (lactone VI 15.11 min) and 16.67 min (lactone VII 16.65 min). Applying the conversion factor calculated from the mean peak area of the direct injections of the standard solution of methyl laurate (14,800 FID area counts/ng), the production rates of the lactones expressed as pg/male/hr were: *C. ferrugineus* lactone I 280 $\pm$ 85 and lactone V 660 $\pm$ 115; *C. pusillus* lactone VI 350 $\pm$ 60, and lactone VII 40 $\pm$ 10.

DISCUSSION

Sexual Differences in EAG Response. Both experiments to investigate the EAG of *C. pusillus* and *C. ferrugineus* to the macrocyclic lactone pheromone components demonstrated significant differences between the EAGs of the sexes, with females giving the larger responses. This contrasts with previous work on the related silvanid beetle *Oryzaephilus surinamensis*, where, although it was possible to demonstrate sexual differences in the behavioral response to macrolide lactone pheromone components, it was not possible to show any differences between the EAGs of the two sexes (White and Chambers, 1989). The larger EAGs of females in *C. pusillus* and *C. ferrugineus* suggests a greater sensitivity to the male-produced aggregation pheromone components, which might be expected if the aggregation pheromone plays any part in mate location and/or courtship (White and Chambers, 1989).

The higher EAG of females might suggest that female antennae possess more acceptor sites of the types involved; however, the receptor numbers in *Cryptolestes* sp. are not known. Male antennae are much longer in *C. pusillus* and slightly longer in *C. ferrugineus*, so the observed sexual difference in EAG may be an artifact resulting from recording from antennae of different lengths. The reason for the sexual dimorphism in antennal length in these cucujid beetles is not clear. Certainly it does not appear to be due to the presence of larger

populations of receptors in the male, at least for receptors of the aggregation pheromone components.

Species Specificity in EAG Response. The EAG of *C. ferrugineus* showed a high degree of specificity for its own aggregation phereomone components, i.e., lactones I and V. The nature of the EAG response does not indicate whether these two components react with the same, or different populations of acceptor sites. In addition, lactones produced by *C. pusillus* or other cucujid or silvanid beetles had little EAG activity, which contrasts with the specificity shown by *C. pusillus*. Although the largest EAGs were elicited by its own aggregation pheromone component VI, both sexes of *C. pusillus* gave consistently large EAGs to lactone V. Significant EAGs also were recorded to the other four lactones. Again, these EAG results cannot show whether there are separate and specific receptors for lactone V and the other mildly active materials, or whether they are interacting with the receptors for lactones VI and VII. Further work on the specificity of the response of single cells is needed to distinguish between these possibilities. The present results do, however, raise the possibility of behavioral cross-attraction between *C. pusillus* and the lactones produced by *C. ferrugineus*, although not vice versa.

Behavioral Responses of Each Species to Its Own Lactones. The first two pitfall bioassay experiments demonstrated clear responses of our strains of *C. ferrugineus* and *C. pusillus* towards the synthetic samples of the chemicals that had been reported to be pheromone components in other strains of these species. With lactones I and V mixed together in equal weights to approximate the ratio reported to be produced naturally (Wong et al., 1983), the response of *C. ferrugineus* increased with dose. Interestingly, the increase was observable both in terms of activity (i.e., an increasing number of beetles responding) and directional response (i.e., an increasing proportion of responders that were found in the pitfall with the lactones). It was also clear that both lactones were effective to some extent individually and could both be contributing to the effect observed with the mixture. For best activity there was nothing to choose between 1000 ng of V, 2000 ng of V, and the mixture of I and V with 1000 ng of each. The best directional response was obtained with either 2000 ng of V or the mixture of I and V with 1000 ng of each. The most effective source combining good activity and direction finding would appear to be one with a mixture of equal proportions of lactones I and V, or the same total weight of lactone V. As a single chemical, the latter would be technically easier to obtain and formulate.

There was a general increase in response of *C. pusillus* to increasing doses of the mixture of lactones VI and VII in the ratio of 20:1, chosen to approximate the ratio previously reported to be produced naturally (Millar et al., 1985). This time, however, maximal activity and directional response were observed before the highest doses had been reached. Again, both lactones were effective individually and were probably contributing to the effect observed with the mix-

ture, although from the results when it was tested alone, the contribution of lactone VII at 100 ng was probably more one of stimulating the activity of the insects rather than attracting them. To get optimal activity and directional response together, a source could probably be composed of either lactone individually at the same weight as the other or a 20:1 mixture of VI and VII at a tenth or even a hundredth of that weight. Thus, in the case of *C. pusillus*, there would be a saving in amount of material needed if both lactones were available.

There are differences between the results obtained in this study and those obtained in previous laboratory pitfall bioassay tests with *C. ferrugineus* (Wong et al., 1983) and *C. pusillus* (Millar et al., 1985). These could arise in part from differences in technique. The conditions used in this study were based on those that had proved successful in our previous work with *O. surinamensis* (White et al., 1989). We used 10-cm-diameter arenas with a filter paper base under low-intensity red light, whereas Oehlschlager et al. (1988), reviewing the work of the Canadian group, quote the use of 15-cm-diameter arenas with roughened plastic bases in the dark. They released 15 insects per replicate in the center of the arena, ran the tests for 2 hr at either 23°C (for *C. ferrugineus*) or 30°C and 60% relative humidity (for *C. pusillus*), and used sexed insects more than once. We used 20 insects per replicate released near the edge of the arena and ran the tests for 1 hr at 25°C and 70% relative humidity, using the insects only once.

These technical points could account for some of the differences in results obtained in this and previous work, but it is unlikely that they would be sufficient to account wholly for the larger discrepancies. For example, lactone VII was reported to elicit no response from *C. pusillus* (Millar et al., 1985) whereas in the present study it was found to be as effective as an equal weight of lactone VI in stimulating both activity and directional response. Differences such as this are more likely to be due to the use of different strains of the two species. In this context it is interesting to note that the strain of *C. pusillus* used by the Canadians had to be reared at less than 1500 insects/kg food and preconditioned before use by aeration in darkness for 48 hr and keeping them in holding vials for 2 hr (Millar et al., 1985). In contrast, the strain used in the present study was reared at 3000 insects/kg and was merely kept for 24 hr without aeration before use. Whether different results between strains are due to differences such as geographical origin or to artifacts such as fitness, method of culturing, or time since collection from the wild, any recommendation to employ a pheromone for practical use would have to include evidence of its effectiveness on more than one strain, and it is encouraging that the present study provides such evidence.

Further differences arise in the treatment of the results. First, in the present study allowance was made for over- or under-dispersion of the data. This effect would, if ignored, lead to incorrect significances being placed on comparisons

of means. In the present study, there were six cases of over-dispersion, one of under-dispersion, and only one where there was no dispersion. This suggests that allowance for dispersion should always be made, or that since it might arise from the insects not behaving independently, tests should only be undertaken with single insects. Another problem with the two-choice pitfall bioassay method is that it combines two effects, namely, whether or not the insects respond and, if they do, which way they go. All previous studies have presented the results in a form that combines these two effects and makes it difficult, if not impossible, to discover the contribution of each individual effect to the overall response. Treating the two effects separately in this study has shown that while some sources elicit both effects, others can differ markedly in their ability to stimulate insects to activity and to respond to a particular pitfall.

Interspecific Behavioral Responses. The result from experiment 5 that *C. pusillus* showed exactly the same directional preference as *C. ferrugineus* for the natural mixture of lactones I and V rather than for an equal total weight of the natural mixture of VI and VII was very surprising but was encouraging from the practical viewpoint, since it might mean that one lure would be suitable for both species. Lest the observation was due to interference between the four lactones, a further experiment was undertaken in which the same sources were tested separately against blanks. This confirmed the greater response of *C. pusillus* towards I and V.

In a review of chemical communication in *Oryzaephilus* and *Cryptolestes* species, Oehlschlager et al. (1988) mentioned that the only species for which cross-attraction has so far been evident is *O. mercator*, which responded to a component of the *O. surinamensis* pheromone (Pierce et al., 1987). However, this probably arises because they share the same enantiomer of this chemical as a component of their individual pheromones [(3*Z*,6*Z*,11*R*)-dodecadien-11-olide, lactone II]. It was also stated that cross-attraction between *C. ferrugineus* and *C. pusillus* was unlikely since the presence of an unique pheromone was considered to be a major mechanism responsible for species specificity (Oehlschlager et al., 1988). However, this prediction has not yet been proven. In fact, it has already been reported that both sexes of *C. pusillus* responded in a suspended disk olfactometer bioassay to a single synthetic component of the male aggregation pheromone of *C. ferrugineus* (Quaife, 1980), although it was not stated which of the two lactones was tested.

Despite the great similarity of the behavioral responses in Figure 5, taxonomic checks confirmed that the species of insect used had been correctly identified. The strain of *C. pusillus* used here might have differed significantly from that studied by Millar et al. (1985) to the extent that its pheromone system might be based on lactones I and V rather than VI and VII. However, the insect aeration experiment showed conclusively that this was not the case. Our strains of both species produced the same lactones as the Canadian strains, even if the

production rates (in pg/beetle/hr) and ratios were different: *C. ferrugineus* our strain, lactone I = 280 and V = 660; Lindgren et al. (1985) I = 640 and V = 990; *C. pusillus* our strain, lactone VI = 350 and VII = 40; Oehlschlager et al. (1988) VI = 1300 and VII = 30. These differences could arise from differences in temperature, age, and population density. The other aeration experiment was undertaken to see if the apparent preference of *C. pusillus* for the mixture of I and V, when presented with a choice between that and an equal weight of the mixture of VI and VII, might be due to a much greater volatility of the former lactones. The results provided no evidence for this, and it must be deduced that the preference of at least this strain of *C. pusillus* for the lactones of *C. ferrugineus* is real if as yet unexplained.

Quaife (1980), reporting the response of *C. pusillus* to a component of the *C. ferrugineus* pheromone, predicted that the two pheromones would prove to be chemically similar, and this has turned out to be true. However, within the set of lactones I–VII discussed here, there are some even closer relationships, e.g., I with II, VI with III, and VII with IV. The interspecific responses found in the present study with *C. pusillus* suggest that it would be worth seeing if there are any for the other species involved.

This study has shown that it might be possible to use single components of each pheromone for trapping the *Cryptolestes* species studied here or to use the *C. ferrugineus* pheromone to attract *C. pusillus*. Moreover, in those cases where mixtures of pheromone components are still necessary, inhibition of attraction of one species to its pheromone due to the presence of the pheromone of another has not yet been found in stored-product coleopterans, so it should be possible to combine pheromones of several species in a single trap (Oehlschlager et al., 1988). This encouraging picture for the use of pheromones to trap stored-product beetles is tempered only by the knowledge that synthetic routes to the lactones at present are still too long and thus improved routes to them are necessary as a matter of of some urgency.

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RAPID, QUANTITATIVE HPLC ANALYSIS OF *Asclepias fruticosa* L.[1] AND *Danaus plexippus* L.[2] CARDENOLIDES

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Abstract—The cardenolide extracts from latex and aerial parts of *Asclepias fruticosa* and of *Danaus plexippus* reared on *A. fruticosa* or *A. curassavica* were purified by adsorption chromatography on silica gel. HPLC analysis on a C_{18} reverse-phase column with an acetonitrile–water gradient as mobile phase, separated 28 compounds with a UV spectrum typical for cardenolides. Afroside and gomphoside (major components), as well as calotropagenin, calotoxin, calotropin, calactin, uscharidin, uscharin, and voruscharin, occurred as single peaks in the profiles of latex and aerial plant parts of *A. fruticosa*. Calactin and calotropin were the major cardenolides in *Danaus plexippus* reared on *A. fruticosa* or *A. curassavica*. Quantitative data obtained with digitoxin as internal standard showed that 1.3–1.5% of the leaf cardenolides were sequestered by *Danaus plexippus* in which levels of 70–80 μg cardenolide per butterfly were measured. The calotropin from the leaves was almost completely sequestered, and 10–13% of the calactin was stored by the butterfly, assuming that no conversion occurred in larval tissues.

Key Words—*Asclepias fruticosa*, milkweed, *Danaus plexippus*, monarch butterfly, Lepidoptera, Danaidae, cardenolides, HPLC, gomphoside, afroside, digitoxin, calactin, calotropin, cardenolide fingerprint, cardiac glycosides, internal standard.

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INTRODUCTION

In the last decades, the levels of cardenolides in various parts of a number of *Asclepias* species have been measured (Roeske et al., 1976). Most of the *Asclepias* species with a high cardenolide content were found to contain a complex mixture, as was shown by TLC, adsorption chromatography, and preparative HPLC. Gomphoside and afroside have been identified as main components in *Asclepias fruticosa* (Carman et al., 1964; Coombe and Watson, 1964; Watson and Wright, 1956, 1957), and recently Cheung et al. (1983) identified 15 cardenolides in this species by [^{1}H]- and [^{13}C]NMR analysis. Nevertheless, quantitative data about cardenolide composition remain scarce. Semiquantitative data were obtained by densitometric analysis or accomplished by visualizing on a subjective but consistent visual 1–5 scale of thin-layer chromatograms (Brower et al., 1982, 1984a,b; Cohen, 1985; Lynch and Martin, 1987; Malcolm et al., 1989; Martin and Lynch, 1988; Nelson et al., 1981; Seiber et al., 1982). Moreover, these data may be affected by unknown losses during extraction and purification procedures of the cardenolide fraction (Brower et al., 1982, 1984a,b; Roeske et al., 1976).

HPLC has been used to separate *Digitalis* and *Nerium* cardenolides. Tittel and Wagner (1981) separated the *Nerium* glycosides on a C_{18} reverse-phase column eluted with a linear gradient of 20–50% acetonitrile in water. A quantitative micro-HPLC analysis of *Digitalis purpurea* cardenolides was performed by Fujii et al. (1983) on an ODS column using a mobile phase of acetonitrile–methanol–water (15:15:19). Desta et al. (1982) reported the isocratic separation of *Digitalis* glycosides on an Ultrasphere C_{18} reverse-phase column with water–methanol–isopropyl alcohol–dichloromethane (47:40:9:4) as mobile phase. The separation of 5α-cardenolides of *Asclepias curassavica* and *A. eriocarpa* was performed on a Bondapak C_{18} reverse-phase column with 30% and 25% acetronitrile in water, respectively, as mobile phase (Benson and Seiber, 1978). Recently, we separated the major cardenolides of *A. curassavica* on a ChromSpher C_{18} reverse-phase column with a methanol–water gradient as mobile phase (Groeneveld et al., 1990).

Substantial improvement in HPLC analysis was obtained with an acetonitrile–water gradient, separating up to 25 cardenolides from *Asclepias fruticosa* latex in a single HPLC run. In this paper we report about a rapid, qualitative and quantitative HPLC analysis of cardenolides from *A. fruticosa* and of *Danaus plexippus* reared on *A. fruticosa* and *A. curassavica*.

METHODS AND MATERIALS

Plants. A. curassavica was grown from seeds in the greenhouse. *A. fruticosa* plants were grown on water culture in a controlled environment (Con-

viron): 13.5 hr light (120–140 μmol/m^2/sec at 125 cm from the light source), temperature 26–28°C; 10.5 hr dark, temperature 20–21°C with an air humidity of 70%. There were four plants in one 3-liter cistern containing a solution of 2.00 mM NO_3^-, 0.27 mM SO_4^{2-}, 0.19 mM H_2PO_4, 0.98 mM K^+, 0.60 mM Ca^{2+}, 0.27 mM Mg^{2+}, 2.00 μM Mn^{2+}, 0.85 μM Zn^{2+}, 0.15 μM Cu^{2+}, 20μM H_3BO_3, 0.25 μM MoO_4^{2-}, 40.0 μM Fe^{2+} in water. *A. fruticosa* was propagated by cuttings.

Butterflies. Eggs from *Danaus plexippus* (probably originating from Costa Rica) were purchased from a commercial butterfly farm. The caterpillars were raised on fresh leaves of either *A. curassavica* or *A. fruticosa*, in an environmental chamber: 14 hr light, temperature 25°C; 10 hr dark, temperature 20°C; air humidity 50–70%. Two-week-old adult butterflies were frozen and stored at −20°C until processed.

Latex (up to 0.5 ml) was collected after incision of the stem with a razor blade and immediately suspended in a 10- to 20-fold volume of a potassium phosphate buffer (0.25 M, pH 7.8). After addition of an equal volume of ethanol 96%, the apolar lipids (mainly triterpene esters) were extracted twice with a few milliliters of light petroleum (40–60°C). The residue was diluted with 4 vol of water and extracted with chloroform (overnight).

The chloroform extracts were dried over anhydrous sodium sulfate and chromatographed on a column of 4 g silica gel G-60 (70–230 mesh; Merck, Darmstadt, Germany), packed in chloroform. The column was eluted stepwise with 30-ml aliquots of chloroform (fraction 1) and chloroform–methanol mixtures of 19 : 1 (fraction 2), 9 : 1 (fraction 3), and 2 : 1 (fraction 4). The fractions were evaporated to dryness under reduced pressure and the temperature did not exceed 50°C. The residue was dissolved in 0.5–1.0 ml methanol and stored at 4°C.

Plant tissue (0.6–4.0 g) was first frozen and then sliced in 1-mm disks (stem) or homogenized in a Waring blender (leaves) and refluxed for 1.5 hr in 20–60 ml ethanol 96%. The ethanol was evaporated under reduced pressure to ca. 10 ml, followed by repeated extraction with light petroleum (40–60°C) until the chlorophyll had been extracted. The residue was diluted with 4 vol of water and extracted with chloroform (overnight). The chloroform extract was dried over anhydrous sodium sulfate and fractionated as described above.

Six samples of two monarch butterflies were homogenized in ethanol 96%, refluxed for 1.5 hr, and extracted with light petroleum and chloroform as described for the aerial plant parts.

The occurrence of cardenolides in the column fractions was determined by the TNDP assay (Brower et al., 1972, 1975). The cardenolide-containing fractions were monitored by TLC on silica gel G-60 (Merck) developed twice in a preequilibrated filter paper-lined glass chamber in ethyl acetate–methanol (97 : 3) (Brower et al., 1982, 1984a,b; Lynch and Martin, 1987; Martin and Lynch, 1988; Nelson et al., 1981; Roeske et al., 1976). Cardenolides were visualized

by spraying with the Kedde reagent: 2% 3,5-dinitrobenzoic acid in methanol followed by 10% sodium hydroxide in 50% methanol (modified after Duffey and Scudder, 1972).

HPLC Analysis. Samples of 5–20 μl (methanol) were injected on a 20 × 0.3-cm ChromSpher C_{18} reverse-phase column with a 5-μm particle size (Chrompack, Middelburg, The Netherlands), equilibrated in acetonitrile–water (27.5% acetonitrile) and eluted with an acetonitrile–water gradient (27.5–70% acetonitrile) with a flow of 0.35 ml/min. The amount of acetonitrile was increased according to the gradient presented in Figure 1, and the eluent was monitored at 220 nm with a Hewlett Packard model 1040A diode array detector. All the peaks obtained were monitored for their UV spectra (200–400 nm). Digitoxin (Merck) was used as internal standard, and this commercially available 5β-cardenolide was added to the ethanol 96% extracts before refluxing: 0.30 mg/g plant tissue (fresh weight), 12.5 μg/butterfly.

RESULTS AND DISCUSSION

All cardenolides from *A. fruticosa* were eluted in the second fraction (chloroform–methanol, 19:1) of the adsorption chromatography. A successful HPLC analysis was achieved with methanol–water gradients (Groeneveld et al., 1990), but peak separation was greatly improved with acetonitrile–water as mobile phase and up to 28 compounds could be eluted in a single run.

A gradient starting with a low-percentage acetonitrile, which was maintained for several minutes, proved to be essential for optimal separation. This percentage appeared to be rather critical: 30% acetonitrile eluted the cardenolides too fast and with many merging peaks; 25% acetonitrile, however, retained the compounds much longer and eluted the first cardenolide after 10 min. A value of 27.5% acetonitrile appeared to be a good compromise (see gradient in Figure 1).

A gradual increase of acetonitrile eluted the cardenolides and the shape of this gradient was adjusted to the eluting compounds by trial and error. Slight variations in the gradient caused drastic shifts of peaks. All the cardenolides in *A. fruticosa* were eluted in 55% acetonitrile. The percentage of acetonitrile was increased to 70% and subsequently maintained for 6 min to elute possible non-cardenolide compounds from the extract. Prior to each injection, 15 min were required to equilibrate the column in 27.5% acetonitrile. The HPLC profile of the latex from *A. fruticosa* (Figure 1, Table 1) contained about 25 peaks eluting within 25 min. All the peaks obtained showed the typical cardenolide UV spectrum as shown by spectrum analysis.

Six cardenolide-containing fractions from *A. fruticosa* latex were obtained in TLC. After partial detection with the Kedde reagent, the scrapings were

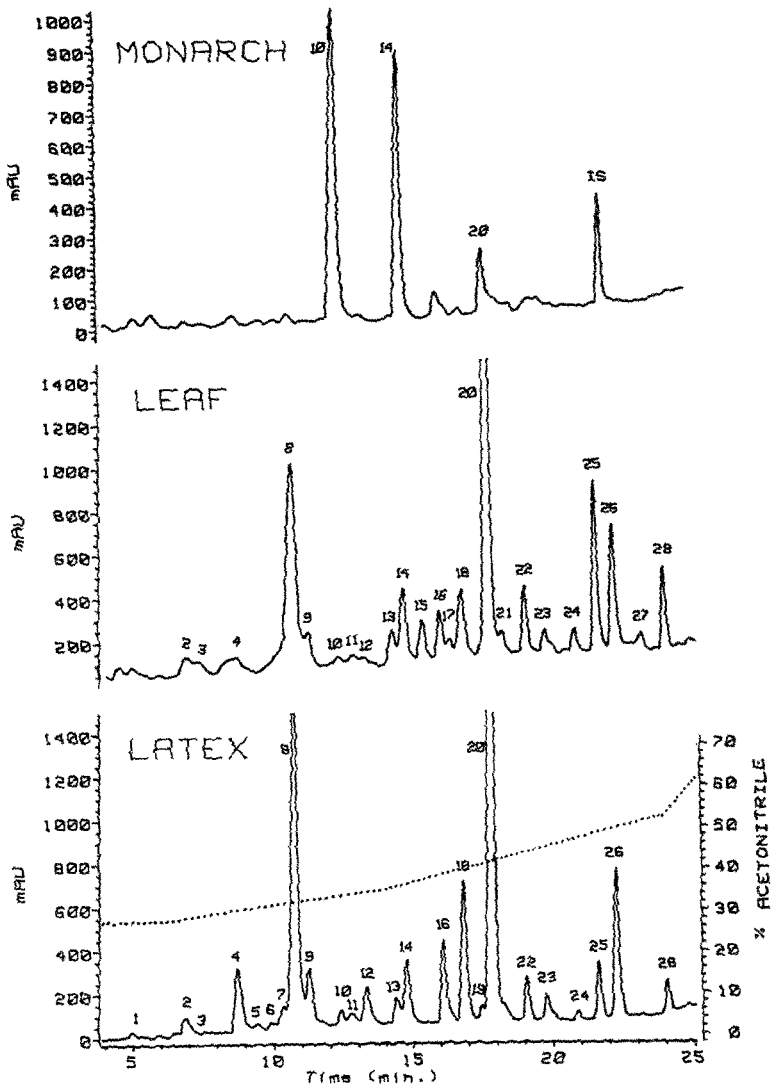


FIG. 1. Reverse-phase HPLC chromatogram of the cardenolides from latex and leaf from *Asclepias fruticosa* and of *Danaus plexippus* reared on this plant. Conditions: Column: 20 × 0.3-cm ChromSpher C_{18} reverse-phase with a 5-μm particle size (Chrompack). Mobile phase: acetonitrile–water, 0.35 *ml/min*. Gradient: 0 min, 27.5%; 6 min, 27.5%; 14 min, 35%; 24 min, 52.5%; 26 min, 70%; 33 min, 70% acetonitrile in water (dotted line). Sample dissolved in methanol: latex 10 μl, leaf 20 μl, butterfly 20 μl. Detection at 220 nm. Peak identities: 1 = calotropagenin; 8 = afroside; 9 = calotoxin; 10 = calotropin; 14 = calactin; 20 = gomphoside; 22 = uscharidin; 25 = uscharin; 26 = voruscharin; IS = internal standard (digitoxin).

TABLE 1. CARDENOLIDE COMPOSITION OF LATEX AND VARIOUS AERIAL PARTS OF *Asclepias fruticosa*

Peak[a]	R_f (min)	Latex	Stem tip	Stem	Leaf 1	Leaf 2	Compound name
1	4.9	0.7	—	41.6	1.3	—	calotropagenin
2	6.8	3.8	15.1	8.6	6.3	2.4	
3	7.5	0.7	3.8	—	33.9	—	
4	8.7	11.7	33.4	9.3	9.2	4.9	
5	9.4	1.9	—	9.8		—	
6	9.9	1.5		—			
7	10.3	3.9					
8	10.7	55.2	141.7	59.4	64.2	35.9	afroside
9	11.3	12.4			9.4	7.8	calotoxin
10	12.4	3.4	6.4	3.5	3.5	1.8	calotropin
11	12.8	3.3	3.8	4.1	3.3	1.5	
12	13.3	8.3	9.3	3.7	2.8	1.1	
13	14.4	5.1	5.4	5.9	6.7	4.8	
14	14.7	10.5	11.7	10.6	15.8	9.6	calactin
15	15.4	—	6.3	—	8.9	2.4	
16	16.1	12.5	13.1	18.3	9.3	8.2	
17	16.4	—	—		3.9	2.8	
18	16.8	19.6	27.3	17.2	16.0	9.7	
19	17.5	2.0	—		—	—	
20	17.8	100.0	100.0	100.0	100.0	100.0	gomphoside
21	18.3	—	5.6	11.7	6.0	3.9	
22	19.1	5.5	11.4	14.3	12.8	17.6	uscharidin
23	19.7	4.2	4.7	2.9	6.5	3.0	
24	20.9	1.0	1.1	2.9	5.0	3.6	
25	21.6	6.2	12.2	8.3	24.9	28.3	uscharin
26	22.2	17.6	12.5	10.7	22.8	10.7	voruscharin
27	23.2	—	—	—	4.0	1.0	
28	24.1	3.0	3.7	5.5	10.1	30.1	

[a] Peak areas are expressed as percent relative to gomphoside (100%). Leaf 1: young growing leaf, leaf 2: expanded (mature) leaf. Data of latex and leaf 2 are from the profiles in Figure 1. Peak areas $<0.5\%$ are omitted. – = not present. Brackets mark merged peaks which could not be integrated separately.

extracted with chloroform–methanol (1 : 1) and the fractions obtained separated in HPLC (Table 2). Comparing these TLC–HPLC data with TLC references in the literature (Brower et al., 1982, 1984a,b; Roeske et al., 1976; Seiber et al., 1983), up to nine compounds in the HPLC profile could be identified. The two major peaks matched the R_{dig} values in TLC reported for gomphoside and afroside in this species. Uscharidin, uscharin, and voruscharin were isolated from the latex of *A. curassavica* (Groeneveld et al., 1990) and matched the mobility of peaks 22, 25, and 26, respectively. Calotropin and calactin were identified

TABLE 2. HPLC COMPOSITION OF SIX TLC SPOTS FROM LATEX CARDENOLIDES[a]

TLC spot	1	2	3	4	5	6	
R_{dig}	1.95	1.79	1.64	1.47	1.21	0.99	
HPLC peak	(%)	(%)	(%)	(%)	(%)	(%)	Compound name
1	—	—	—	—	—	29.1	calotropagenin
2	—	—	—	—	2.1	8.1	
3	—	—	—	—	1.4	—	
4	7.5	6.1	1.1	2.7	56.0	—	
5	—	—	—	0.3	1.5	—	
6	—	6.9	—	—	—	—	
7	—	—	—	—	—	12.8	
8	—	—	4.1	44.0	3.2	—	afroside
9	—	—	—	—	18.4	50.0	calotoxin
10	—	—	—	1.5	10.0	—	calotropin
11	—	—	—	—	3.7	—	
12	—	2.5	12.4	1.0	—	—	
13	—	10.2	—	—	—	—	
14	—	—	3.6	11.7	—	—	calactin
15	—	—	—	—	—	—	
16	11.4	2.7	—	16.2	—	—	
17	—	—	—	—	—	—	
18	—	40.7	2.6	0.5	—	—	
19	12.3	—	—	—	—	—	
20	7.0	9.1	74.8	9.9	3.7	—	gomphoside
21	—	2.0	—	—	—	—	
22	—	—	—	4.0	—	—	uscharidin
23	6.6	10.7	—	—	—	—	
24	3.5	—	—	—	—	—	
25	—	—	—	5.2	—	—	uscharin
26	51.7	9.1	—	—	—	—	voruscharin
27	—	—	—	—	—	—	
28	—	—	1.4	3.0	—	—	
	100.0	100.0	100.0	100.0	100.0	100.0	

[a]HPLC peak numbers refer to the peak numbers in Figure 1. (The minor peaks 15, 17, and 27 do not occur in the latex.) — = not present.

by comparison with the TLC data of the major cardenolides of *Danaus plexippus* reared on *A. fruticosa*. Calotropagenin and calotoxin were tentatively identified by comparison with TLC data only. Cheung et al. (1983) reported 15 different cardenolides for *A. fruticosa*, but our profile indicates at least 10 more compounds to be identified. Several peaks (e.g., 4 and 16) may be composed of two or more compounds, as these peaks occur in various spots obtained by

TLC (Table 2). In addition, a few small peaks had shoulders, which indicates merged peaks.

In contrast with the colorless cardenolide extracts obtained from latex, the extracts from stem and, in particular, from the leaves had a yellow color. The colored compounds, however, were not eluted in the programmed gradient and did not interfere with the cardenolide profile (100% acetonitrile eliminated these colored compounds from the column). Nearly all the compounds detected in the latex were present in stem and leaf, but their mutual ratios varied (Table 1).

As no typical major latex cardenolide could be assigned, the latex contribution to the cardenolides in stem and leaf could not be calculated as was possible in *A. curassavica* (Groeneveld et al., 1990). Results in Table 1 indicate a rather stable cardenolide composition throughout the plant. The major components gomphoside and afroside accounted for 42–56% of the cardenolide in the various tissues. Gomphoside was the major component in most tissues. Afroside occurred as the major constituent in the stem tip, but appeared as a broadened peak in the profile and undoubtedly contains other minor glycosides. Peak broadening occurred frequently in the 5- to 10-min area of the profile after injection of 20-μl samples: the minor peaks 5, 6, and 7 (occurring in the latex profile) merged with peak 8 in the leaf and stem extracts (Figure 1; Table 1).

The commercially available 5β-cardenolides digitoxin and digitoxigenin were eluted with the *Asclepias* cardenolides in fraction 2 in adsorption chromatography. Digitoxigenin cochromatographed in HPLC with the major component gomphoside and could therefore not be used as internal standard. Digitoxin appeared later in the profile and eluted in between peaks 25 and 26 (Figure 1). As both the 5α-cardenolides and digitoxin were detected by their typical UV absorption maximum at 220 nm of the butenolide ring, the composition of an extract could be quantified in micromoles. Leaves of *A. fruticosa* grown in a controlled environment contained 1.1 ± 0.05 μmol/g cardenolides.

The cardenolide extracts of the monarch butterflies reared on *A. fruticosa* or *A. curassavica* revealed two major components in HPLC (Figure 1) besides a number of minor components with a typical cardenolide UV spectrum. Calotropin and calactin have been reported as main constituents by Benson and Seiber (1978) and by Cheung et al. (1988) for *Danaus plexippus* reared on *A. curassavica* and *A. fruticosa*, respectively, and compounds 10 and 14 from the butterfly matched the corresponding R_{dig} values in TLC. The profiles of butterflies reared on *A. fruticosa* contained a small peak that cochromatographed with the gomphoside. Voruscharin and uscharin did not occur in the chloroform extracts of *Danaus plexippus*. Using digitoxin as an internal standard, 70–80 μg of cardenolides per butterfly were assayed, which is in the lower range of data presented in the literature (Brower et al., 1982, 1984a,b; Cohen, 1985;

Lynch and Martin, 1987; Malcolm et al., 1989; Martin and Lynch, 1988; Roeske et al., 1976).

The butterflies reared on *A. fruticosa* leaves contained 39 μg calotropin and 26 μg calactin per butterfly. The caterpillar consumed 8–10 g leaf tissue of this plant species. The occurrence of individual cardenolides was calculated by combining the data of Table 1 with a cardenolide content of 1.1 μmol/g leaf. Assuming that peaks 10 and 14 do not contain other cardenolides, 37–54 μg calotropin and 199–246 μg calactin were available per caterpillar. These data, then, indicate that calotropin can be almost completely sequestered and that 10–13% of the available calactin was finally stored by the butterfly, assuming that no conversions take place in larval tissues. If these conversions do occur, it can be concluded that about 1.3–1.5% of the consumed cardenolides were sequestered by *Danaus plexippus* reared on *A. fruticosa*.

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(+)-JUNIPEROL AND (+)-PIMARAL: ATTRACTANTS FOR THE CERAMBYCID BEETLE, *Monochamus alternatus* HOPE

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Abstract—A sesquiterpene and a diterpene were isolated from sound pines and identified as (+)-juniperol and (+)-pimaral, respectively. The combination of these compounds in a certain ratio induced a significant laboratory flight response by the female cerambycid beetle, *Monochamus alternatus* Hope. Individual compounds elicited a limited response only.

Key Words—*Monochamus alternatus* Hope, Coleoptera, Cerambycidae, female, *Pinus densiflora*, *Pinus thunbergii*, attractants, (+)-juniperol, (+)-pimaral, sesquiterpenes, diterpenes.

INTRODUCTION

The cerambycid beetle, *Monochamus alternatus* Hope is a vector of the pine wood nematode, *Bursaphelenchus xylophilus* Steiner et Buhrer, which enters a pine through the feeding wound formed by the beetle (Mortimoto and Iwasaki, 1972; Mamiya and Enda, 1972). Pine forests are devastated by the combination of the beetle and the nematode. As sites for oviposition, the beetle favors oleoresin-exuding (Iwasaki and Morimoto, 1970), felled (Iwasaki and Morimoto, 1973), nematode-infested (Morimoto and Iwasaki, 1972), and paraquat-treated (Yamasaki et al., 1980) pines. The attraction of a nematode-infested pine for the beetle is caused by a synergistic effect of ethanol produced in the diseased tissues and inherent monoterpene hydrocarbons (Ikeda, et al., 1980 a,b, 1986). Attractants had been isolated from volatiles in paraquat-induced lightwood using

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a walkway olfactometer without chemical identification (Sakai and Yamasaki, 1989).

It has been believed that sound pines are not selected as sites favorable for oviposition by the beetle because of the "absence" of attractants. However, the presence of oviposition stimulants in the pine inner bark was found recently (Yamasaki et al., 1989). An unknown factor therefore must be present, which is responsible for a negative selection as an oviposition site. The present communication deals with the isolation and the structure identification of the attractants from sound pines.

METHODS AND MATERIALS

Instrumental Analysis. Mass (MS) spectra were measured with a JEOL JMS D-300 mass spectrometer with electron ion at 70 eV. High-resolution (HR) MS spectra also were measured with the instrument. Proton nuclear magnetic resonance ([^{1}H] NMR) spectra were run at 500 MHz on a JEOL JNM-GSX 500 spectrometer. Carbon-13 ([^{13}C]) NMR sprectra were run at 67.8 MHz on a JEOL GX-270 spectrometer. [^{13}C–^{1}H] shift correlation spectroscopy (COSY)-, [^{1}H–^{1}H]COSY-, and nuclear Overhauser effect (NOE) difference spectra were taken with the JNM-GSX 500 spectrometer. Multiple-bond [^{13}C–^{1}H]COSY spectra were taken with the GX-270 spectrometer. All NMR spectra were obtained at 27.0°C using deuterochloroform solutions with tetramethylsilane as an internal standard. Optical rotation ($[\alpha]_D$) was measured with a Horiba SEPA-200 polarimeter.

Isolation of Bioactive Compounds. Sound 12- to 15-year-old *Pinus desiflora* Sieb. et Zucc. and *P. thungergii* Parl. were harvested in mid-May 1989 in a stand where no mortality of pines was caused by the nematode. No feeding wound formed by the beetle was found on the branches. No nematode was detected in either the phloem or the xylem by use of the Baermann funnel technique.

Essential oils were obtained by steam distillation from freshly crushed entire trunks of the two species, *P. densiflora* and *P. tunbergii*, i.e., 21.3 g and 33.7 g of essential oils, respectively. The oils were distilled up to 51° at 1.5 mm Hg. The residues were subjected to low-temperature liquid chromatography (Yamasaki et al., 1986; Sakai and Yamasaki, 1988), to separate a fraction of minor constituents (diethyl ether eluate) from the others (*n*-pentane eluate). The essential oils from *P. densiflora* and *P. thunbergii* contained 1.6% and 1.8% minor constituent fractions, respectively.

The minor constituents were subdivided by silica gel thin-layer chromatography (TLC) using *n*-pentane–ether (5:1). A range of fractions giving R_f values of 0.23–0.61 was gas chromatographed on a 3-mm-ID × 2-m glass col-

umn (50–200°C) packed with 10% polyethylene glycol 20M at a program rate of 4°C/min. Compounds **1** from *P. densiflora* and **1a** from *P. thunbergii* giving a relative retention value of 1.80 (longifolene = 1.00) and compounds **2** from the former and **2a** from the latter giving that of 2.24 were collected in cold traps and purified by TLC.

The Insect. Approximately 100 females of *M. alternatus* (both sexes being attracted to paraquat-treated pines) were collected during mid-July 1989 (Yamasaki and Suzuki, 1982). They were marked with white spot(s) to distinguish individuals (Yamasaki and Sunago, 1983), and kept in individual glass bottles (Yamasaki and Suzuki, 1982).

Attractant Bioassay. A chamber for bioassay was an entire room (6.8 × 2.6 × 3.8 m) without an interfering factor (Figure 1). The room was 2.5 m away from an open doorway. As air inlets, individual polyvinyl chloride pipes (15 cm ID × 90 cm) were set into one side (3.8 m wide) at each point 95 cm distant of its ends 2.1 m above the floor. Individual hardware cloth screens (30 × 30 cm), necessary for responding beetles to land on, were fixed to one end from each pipe. To prevent sample-impregnated filter paper from being blown around, each pipe was partitioned with hardware cloth 35 cm distant from the other end. To emit odor outdoors, a fan was used which was set into the opposite side 2.3 m above the floor. A wooden duct with a 35 × 35-cm section was fixed to the fan. The duct intake was covered with hardware cloth, and kept 55 cm distant from the floor. All apertures in six sides of the room were taped.

Twenty-five females were placed on the floor near the duct intake at 7:30 PM. A ventilation rate of approximately 5.1 m^3/min was maintained. Bioassays of 1 hr were conducted at 30-min intervals from 8:00 PM to 1:30 AM. A piece of filter paper impregnated with an *n*-pentane solution of a sample was placed in a Petri dish near the partition inside one of the inlets. Real and total numbers of individual females that landed on the screen on the odor and the air (blank) inlets were counted.

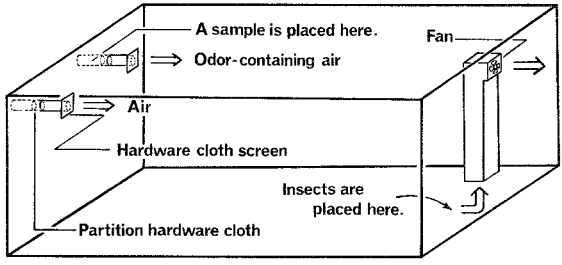


FIG. 1. Diagram of a chamber type olfactometer for *M. alternatus* (drawn to scale).

At the 30-min intervals, the ventilation rate was increased to approximately 12.3 m^3/min. Every night, 25 females were randomized.

RESULTS

Attraction of Minor Constituent Fraction for M. alternatus. When ventilation was conducted without delivering any sample, most insects were at rest or walking, especially on the leeward. A few were flying, but no female was observed landing on either screen. In contrast to such behavior on the blanks, shortly after a piece of filter paper containing minor constituent fraction from *P. densiflora* was set in one of the inlets, some insects responded to the odor source while ranging from one point to another or turning in the air. Females that landed near the odor source did not walk to it.

Of the different amounts tested (Table 1), 240 μg of the minor constituent fraction elicited a maximum flight response: on average 3.7 females on 16 min (T_1), i.e., 11 in three replications. Either more or less than 240 μg of the fraction reduced the flight response. Only one female was observed landing on the

TABLE 1. NUMBERS OF *M. alternatus* FEMALE ATTRACTED TO MINOR CONSTITUENT FRACTION FROM SOUND *P. densiflora* (JULY, 27 TO 31, 1989)

Treatment	Amount of the fraction (μg)[a]	Real No.	Total No.	Time elapsed (min)[b]		
				T_1	T_2	T_3
Minor constituent fraction	900	3.0(2-4) [0.3(0-1)]	3.7(3-4) [0.3(0-1)]	17(15-18) [14(14)]	19(19-20) [14(14)]	21(20-23) [15(15)]
	480	3.3(3-4) [0]	4.3(4-5) [0]	17(15-18)	19(18-21)	21(19-23)
	240	3.7(3-5) [0]	5.0(4-6) [0]	16(14-18)	18(17-20)	20(20-21)
	120	1.0(1) [0]	1.0(1) [0]	16(15-16)	16(15-16)	19(18-20)
	30	0.7(0-1) [0]	0.7(0-1) [0]	14(12-15)	14(12-15)	17(15-18)
n-Pentane	0	0 [0]	0 [0]			

[a] A 40 μl *n*-pentane solution of the fraction or a 40 μl *n*-pentane was presented only at the onset.
[b] T_1, from start to time when real number reached the maximum shown above; T_2, to time when total number reached the maximum; T_3, to time when the last individual flew away. Number and time represent the mean of three replications (range in parentheses). Blanks are shown in brackets.

blank screen in three replications when 900 μg of the fraction was presented. On the average, after 17–21 min (T_3), no female was observed on either screen; after that, the females responded as they did to the blanks. The solvent elicited no flight response.

Identification of Bioactive Compounds. For convenience, signals in [^{1}H]- and [^{13}C]NMR spectra were marked with letters in the alphabet and Arabic numerals, respectively. The kinds of carbons were determined by NMR distortionless enhancement by polarization transfer (DEPT).

Compound 1. MS: *m/z* (%) 222(M^+, 8.6), 207(47), 204(91), 189(75), 161(47), 135(22), 121(25), 119(90), 109(58), 95(95), 85(100), 69(36). HR-MS: found: 222.1974, calcd. for $C_{15}H_{26}O$; 222.1983. [^{1}H]NMR: δ 0.833(3Ha, s), 0.859(3Hb, s), 0.930(3Hc, s), 0.934(3Hd, s), 0.964(1He, d, J = 4.9Hz), 1.133–1.172(1Hf, m), 1.180–1.189(1Hg, m), 1.252–1.301(1Hh, m), 1.330–1.358(3Hi, m), 1.411–1.464(2Hj, m), *ca.* 1.51–1.61(-OH, broad), 1.701–1.719(1Hk, m), 1.836(1Hl, d, J = 4.6Hz), 1.852–1.897(1Hm, m), 3.757(1Hn, dd, J = 2.1, 4.9Hz). [^{13}C]NMR: δ 12.99(C1, $-CH_3$), 22.54(C2, $>CH_2$), 22.68(C3, $-CH_3$), 26.39(C4, $>CH_2$), 28.92(C5, $-CH_3$), 29.25(C6, $-CH_3$), 30.34(C7, $>CH_2$), 33.42(C8, $>C<$), 35.21(C9, $>CH_2$), 41.07(C10, $>CH_2$), 44.13(C11, $>CH-$), 50.24(C12, $>C<$), 51.33(C13, $>C<$), 64.66(C14, $>CH-$), 79.77 (C15, $>CH-$). $[\alpha]_D^{24}$= +20.5° (c = 0.3, EtOH).

Compound 2. MS: *m/z* (%) 286(M^+, 30), 271(46), 257(100), 187(15), 175(14), 161(29), 147(27), 135(41), 133(38), 123(87), 121(56), 119(37), 117(18), 115(7), 109(40), 107(65), 105(42), 97(22), 95(34), 93(54), 91(56), 81(65), 79(50), 77(24), 67(40), 55(58). HR-MS: found: 286.2304, calcd. for $C_{20}H_{30}O$; 286.2297. [^{1}H]NMR: δ 0.797(3Ha, s), 0.998(3Hb, s), 1.024–1.080(2Hc, m), 1.087(3Hd, s), 1.208–1.266(1He, m), 1.269–1.393(2Hf, m), 1.405–1.493(1Hg, m), 1.508–1.550(2Hh, m), 1.555–1.609(4Hi, m), 1.723(1Hj, dd, J = 1.5, 12.5Hz), 1.798(1Hk, t, J = 8.3Hz), 2.103(1Hl, dt, J = 5.4, 13.7Hz), 2.279(1Hm, ddd, J = 2.0, 4.6, 14.3Hz), 4.919(1Hn, dd, J = 2.0, 17.7Hz), 4.947(1Ho, dd, J = 2.0, 10.7Hz), 5.160(1Hp, d, J = 1.5Hz) 5.716(1Hq, dd, J = 10.5, 17.3Hz), 9.242 (1Hr, s). [^{13}C]NMR: δ 14.69(C1, $-CH_3$), 15.18(C2, $-CH_3$), 17.31(C3, $>CH_2$), 18.99(C4, $>CH_2$), 24.61(C5, $>CH_2$), 29.41(C6, $-CH_3$), 32.54(C7, $>CH_2$), 35.25(C8, $>CH_2$), 35.59 (C9, $>CH_2$), 37.26(C10, $>C<$), 38.21(C11, $>CH_2$), 38.62(C12, $>C<$), 46.77(C13, $>CH-$), 49.70(C14, $>C<$), 51.26(C15, $>CH-$), 112.86(C16, $>CH_2$), 128.82(C17, $>CH-$), 137.57(C18, $>C<$), 147.21(C19, $>CH-$), 206.51(C20, $>CH-$). $[\alpha]_D^{24}$ = +4.99° (c = 0.3, EtOH).

Protons linked directly to individual carbons of these compounds were specified by [^{13}C–^{1}H]COSY. Protons on second and third carbons starting from the individual carbons were disclosed by multiple-bond [^{13}C–^{1}H]COSY. From these data, the second or third carbons were explored. The protons on the second carbons were specified using [^{1}H–^{1}H]COSY. The third carbons were deduced. Thus, all C-H and C-C connectivities were clarified.

The protons were revealed that gave rise to their respective NOEs as a result of irradiating some protons. Thus the configurations of key carbons (C8, C11, C13, and C15 of compound **1** and C10, C12, C13, C14, and C15 of compound **2**) were proved.

Thus, compounds **1** and **2** were identified as (1*R*, 3aR, 4*S*, 8a*S*, 9*S*)-(+)-decahydro-1,5,5,8a-tetramethyl-1,4-methanoazulen-9-ol [(+)-juniperol] and (1*R*, 4a*R*, 4b*R*, 7*S*, 10a*R*)-(+)-1,2,3,4,4a,5,6,7,9,10,10a-dodecahydro-1,4a,7-trimethyl-7-vinyl-1-phenanthrencarbaldehyde [(+)]-pimaral], respectively (Scheme 1). Previous [^{1}H]NMR spectral data of synthetic (+)-juniperol (Kuo and Money, 1986) and other data of synthetic (+)-pimaral (Wenkert et al., 1965), support the identification of compounds **1** and **2**, respectively.

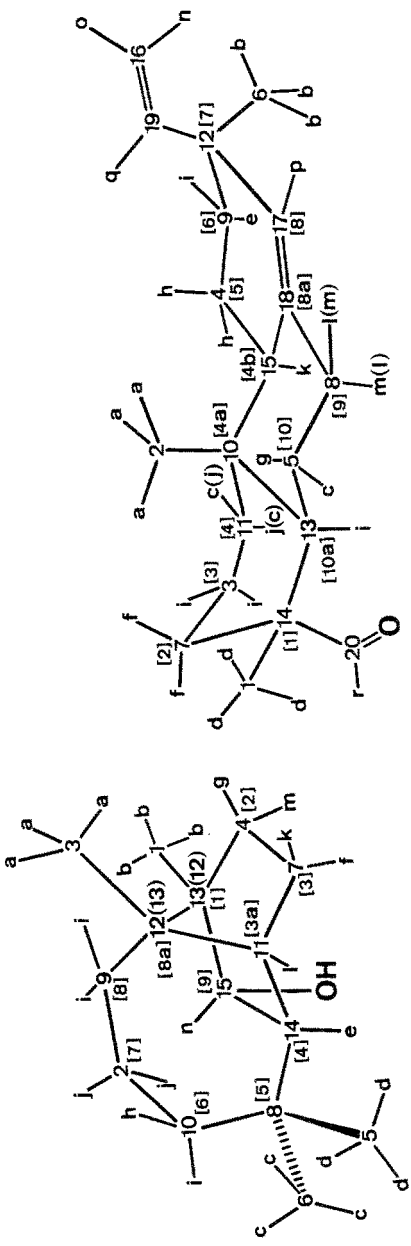
MS, HR—MS, [^{1}H]NMR, [^{13}C]NMR, and NOE data of compounds **1a** {$[\alpha]_D^{24}$ was +17.7° (c = 0.3, EtOH)} and **2a** {$[\alpha]_D^{24}$ was +7.33° (c = 0.3, EtOH)} coincided with those of the (+)-juniperol and the (+)-pimaral, respectively. In the same way, both compounds were found to be present in paraquat-induced lightwood materials that also exhibit a high activity for the beetle.

Attraction of (+)-Juniperol and (+)-Pimaral for M. alternatus. It was confirmed by capillary-gas chromatographic analysis that 240 μg of the minor constituent fraction from *P. densiflora* contained 10.3 μg of (+)-juniperol and 29.7 μg of (+)-pimaral, and the same amount of that from *P. thunbergii* contained 14.9 μg of (+)-juniperol and 17.2 μg of (+)-pimaral.

Table 2 shows the attraction capabilities of such quantities of the compounds for the female. In the first successful experiment (August 1), the first blend of (+)-juniperol and (+)-pimaral from sound *P. densiflora* attracted seven females. Such attraction capability of the binary blend appeared to be almost equivalent to that of the minor constituent fraction. On the other hand, the individual compounds attracted only two or three females. After the test sample and blank sides were interchanged, the individual compounds and the blend were again bioassayed in a different order (August 2). The females were highly responsive to both the blend and the fraction. The second blend of (+)-juniperol and (+)-pimaral from sound *P. thunbergii* also was found to be highly attractive (August 3). The third blend, prepared with reduced quantities, attracted only two females (August 3). There was no solvent response.

DISCUSSION

Tables 1 and 2 indicate clearly that (+)-juniperol and (+)-pimaral isolated from sound *P. densiflora* and *P. thunbergii* are attractants for *M. alternatus* female. Previous investigations dealt with isolations of (+)-juniperol from some families of conifers, e.g., *Juniperus communis* L. (Mattsson, 1913) growing in Finland, *Cupressus macrocarpa* Hartw. (Briggs and Sutherland, 1942) in the



SCHEME 1. (+)-Juniperol (left) and (+)-pimaral (right). Individual carbons and protons labeled with Arabic numerals and letters of the alphabet, respectively, to reflect structure analysis used here. Carbon numbers based on the IUPAC rules are shown in brackets.

TABLE 2. NUMBERS OF *M. alternatus* FEMALE ATTRACTED TO (+)-JUNIPEROL(J) AND (+)-PIMARAL(P) PRESENTED INDIVIDUALLY AND IN COMBINATION (1989)

	August 1			August 2			August 3		
Time period[a]	Treatment I[b]	Real No.	Total No.	Treatment II[b]	Real No.	Total No.	Treatment III	Real No.	Total No.
A	J(10.3)	3[0]	5[0]	J + P (10.3 vs. 29.7)	9[0]	13[0]	$J^b + P^b$ (1.30 vs. 3.74)	2[0]	2[0]
B	P(29.7)	2[0]	3[0]	Minor constituent fraction (240)	10[0]	14[0]	*n*-pentane (40 μl)	0[0]	0[0]
C	J + P (10.3 vs. 29.7)	7[1]	15[1]	P(29.7)	3[0]	3[0]	$J^c + P^c$ (14.9 vs. 17.2)	8[1]	16[1]
D	Minor constituent farction (240)	8[1]	13[1]	J(10.3)	3[0]	4[0]	Minor constituent fraction[c] (240)	8[1]	14[1]

[a] A, 8:00 to 9:00 PM; B, 9:30 to 10:30 PM; C, 11:00 to 0:00 AM; D, 0:30 to 1:30 AM.
[b] With the samples from *P. densiflora*.
[c] From *P. thunbergii*. *n*-Pentane solutions (40 μl each) containing the amount (μg) shown in parentheses were presented at 15-minute intervals. Blanks are shown in blackets.

United States, *Dacrydium cupressinum* Solander (Brandt and Thomas, 1951) in New Zealand, and *P. thunbergii* (Akiyoshi et al., 1960) in Japan. (+)-Pimaral was identified in the pine genus, e.g., *P. sylvestris* L. in Norway (Sörensen and Bruun, 1947) and the United States (Harris and Sanderson, 1948), *P. elliottii* Engelm. and *P. palustris* Mill. (Harris and Sanderson, 1948) in the United States, and *P. contorta* Dougl. (Manning, 1973) in New Zealand.

However, to our knowledge, (+)-juniperol and (+)-pimaral have not been identified as attractants for any species. The identification of these compounds as attractants for the *M. alternatus* female indicates the possibility that the beetle may select sound pines as sites for oviposition when other favorable sites are in short supply or absent. It also is suggested that sound pines may emanate unknowns that could prevent oviposition preference by *M. alternatus*.

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IDENTIFICATION AND FIELD TESTING OF FEMALE-PRODUCED SEX PHEROMONE COMPONENTS OF THE SPRING CANKERWORM, *Paleacrita vernata* PECK (LEPIDOPTERA: GEOMETRIDAE)[1]

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Abstract—Two sex pheromone components, 3(Z),6(Z),9(Z)-nonadecatriene (3Z,6Z,9Z-19:H), and 3(Z),6(Z),9(Z)-eicosatriene (3Z,6Z,9Z-20:H), have been positively identified, and a third component, 6(Z),9(Z)-nonadecadiene (6(Z),9(Z)-19:H) has been tentatively identified from abdominal tip extracts of female spring cankerworm moths, *Paleacrita vernata* Peck (Lepidoptera: Geometridae). The pheromone components were identified by a combination of gas chromatography, electroantennography, mass spectrometry, chemical tests, comparison with standards, and field testing. Only 3Z,6Z,9Z-20:H exhibited significant attractant activity when tested alone, and it was potentiated by the other two components. The attractive blend was an 8:2:1 ratio of 3Z,6Z,9Z-20:H/3Z,6Z,9Z-19:H/6Z,9Z-19:H. However, the two-component blend of 3Z,6Z,9Z-20:H and 6Z,9Z-19:H (8:1 ratio) was as attractive as the three-component blend in further field tests. A series of related compounds, the diene monoepoxides available from epoxidation of C_{19} and C_{20} 3Z,6Z,9Z-trienes, some of which have been found in the pheromone blends of other moth species, were tested as behavioral antagonists. The attraction of male moths to synthetic lures was suppressed by the addition of

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6Z,9Z-*cis*-3,4-epoxy-nonadecadiene to the lures. Additional experiments were performed to determine the effects of lure dosage, trap height, and trap design on the numbers of male moths captured.

Key Words—Pheromone, attractant, 6(Z),9(Z)-nonadecadiene, 3(Z),6(Z),9(Z)-nonadecatriene, 3(Z),6(Z),9(Z)-eicosatriene, 6(Z),9(Z)-*cis*-3,4-epoxy-nonadecadiene, *Paleacrita vernata*, spring cankerworm, Lepidoptera, Geometridae, trap height, behavioral antagonist.

INTRODUCTION

The spring cankerworm, *Paleacrita vernata* Peck (Lepidoptera: Geometridae), is a very common pest in orchards, shade trees, and shelter belts in Canada and the United States. Outbreaks can result in serious defoliation and continued attacks can cause serious damage to trees (Furniss and Carolin, 1977; Ives and Wong, 1988).

The adult insects emerge from pupation chambers in the soil very early in the spring, often when there is still residual snow cover. Females are wingless, and upon emergence, climb nearby vertical surfaces to call and mate. Eggs are commonly laid in bark crevices. The larvae emerge at about the time of bud swelling in May, develop on the foliage, and then drop to the ground, where they construct cells in the soil in which they overwinter. Pupation occurs in late winter, completing the life cycle (Ives and Wong, 1988).

As a section of a larger project involving the characterization of unsaturated hydrocarbon and epoxide sex pheromones of geometrid and noctuid moths, we report here the identification and field testing of 3(Z),6(Z),9(Z)-nonadecadiene (3Z,6Z,9Z–19:H; subsequent abbreviations follow the same pattern), 6Z,9Z–19:H, and 3Z,6Z,9Z–20:H as female-produced sex pheromone components of the spring cankerworm.

There is a growing body of literature describing these types of compounds and related epoxides, tetraenes, and ketones as sex attractants and sex pheromones for a number of arctiid, geometrid, noctuid, and amatid moth species. For example, a number of species are attracted by 3Z,6Z,9Z-trienes of chain lengths C_{17} to C_{23}, either as single components or in blends (Heath et al., 1983; Landolt et al., 1986; McLaughlin and Heath, 1989; Millar et al., 1987,1990a–c; Szöcs et al., 1987; Underhill et al., 1983; Wong et al., 1985). Several species are known to use blends of 3Z,6Z,9Z-trienes with 6Z,9Z-dienes (Bell and Meinwald, 1986; Bögenschutz et al., 1985; Descoins et al., 1986; Jain et al., 1983) or tetraenes (1,3(Z),6(Z),9(Z)-heneicosatetraene, 1,3Z,6Z,9Z–21:H; Jain et al., 1983; 3Z,6Z,9Z, 11E–19:H and 3Z,6Z,9Z,11Z–19:H, Wong et al., 1984). Several species apparently utilize 1,3Z,6Z,9Z-tetraenes as single-com-

ponent pheromones (Underhill et al., 1987; Roelofs et al., 1982; Bestman et al., 1982; Szöcs et al., 1987).

A considerably larger number of species is attracted to blends of 3*Z*,6*Z*,9*Z*-trienes with one or more *cis*-epoxydienes, with the epoxides being in the 3,4; 6,7; or 9,10 positions (Becker et al., 1987; Bell and Meinwald, 1986; Descoins and Frérot, 1984; Millar et al., 1987, 1990a–c; Wong et al., 1985). Di- and triunsaturated aldehyde pheromone components with similar types of methylene interrupted double-bond systems have been reported in blends with a diunsaturated epoxide from two arctiid species (Hill and Roelofs, 1981; Hill et al., 1982). There is a single reported example of a ketone (6*Z*,9*Z*-3-one-19 : H) in combination with 3*Z*,6*Z*,9*Z*-19 : H as the pheromone blend of a geometrid moth (Buser et al., 1985), and one example of epoxytriene pheromone components (1,3*Z*,6*Z*-9*S*,10*R*-epoxy-20- and 21 : H, Tóth et al., 1989).

There also has been one report of a monoene epoxide (6*Z*-9*S*,10*R*-epoxy-21 : H), occurring in a pheromone blend in combination with 6*Z*,9*Z*-21 : H, 3*Z*,6*Z*,9*Z*-21 : H, and 3*Z*,6*Z*-*cis*-9,10-epoxy-21 : H (Descoins and Frérot, 1984), and several other examples of monoene epoxides as lepidopteran sex attractants have been discovered (Millar and Underhill, unpublished data).

We have been particularly interested in identifying pheromones from moth species with wingless females, as these species may be well suited to control by mating disruption. That is, females are of limited mobility, and thus the problem of mated females moving into a pheromone treated area from a non-disrupted area will be minimized. We report here: (1) the details of the identification of the female-produced sex pheromone components of the spring cankerworm by a combination of electroantennography (EAG), coupled gas chromatography–electroantennogram detection (GC-EAD), gas chromatography–mass spectrometry (GC-MS), and chemical tests; (2) the results of field testing of synthetic sex pheromone components; (3) the results of field testing of potential behavioral antagonists; (4) the efficacy of various trap designs for capturing male spring cankerworm moths; and (5) the effect of trap height on captures of male spring cankerworm moths.

METHODS AND MATERIALS

Insects and Electroantennography. Female moths were collected by hand each morning as they climbed the trunks of trees in a shelter belt composed of Siberian elms (*Ulmus pimula*). Male moths were taken on sticky bands wrapped around the trunks of trees at a height of approx. 1 m, in light traps, or in sticky traps baited with synthetic sex attractants or female insects. Male antennal responses to extracts of females or to synthetic chemicals were recorded as previously described (Millar et al., 1987). Synthetic compounds tested in EAG

studies included (chain lengths C_{18-22} unless otherwise specified): saturated hydrocarbons, monounsaturated hydrocarbons, 6Z,9Z-dienes, 3Z,6Z,9Z-trienes and the monoepoxides derived therefrom, 6Z,9Z,12Z-trienes, 6Z,9Z,12Z,15Z-tetraenes, 9Z,12Z,15Z-octadecatriene alcohol, acetate, and aldehyde, and 1,3Z,6Z,9Z-nonadecatetraene. Compounds tested in GC-EAD studies included (chain lengths C_{18-22}) 6Z,9Z-dienes, 3Z,6Z,9Z-trienes and the monoepoxides derived therefrom.

Extracts of sex pheromone glands were made by immersion of excised female ovipositors in methylene chloride for 20 min. Extracts were concentrated under a stream of nitrogen. For GC-EAD and GC-MS runs, internal standards (heptadecane and tetracosane, unless otherwise specified) were added. GC-MS analyses were carried out with a Finnegan 4000E instrument interfaced to an Incos 2300 data system, in electron impact (70 eV) or chemical ionization (methane) modes. Capillary columns (50 m × 0.32 mm) coated with DB-5 (J&W Scientific, Folsom, California) or Ultra-2 (Hewlett-Packard, Avondale, Pennsylvania) were used with helium carrier gas, programming from 40 to 250°C. GC-EAD studies were carried out as previously described (Millar et al., 1987).

Insect Trapping. Field experiments were carried out in windbreaks in Saskatoon or Indian Head, Saskatchewan. Pherocon 1CP wing traps [Scentry Inc., (formerly Zoecon, Inc.), Buckeye, Arizona] or the equivalent were used throughout, except for the experiment testing trap design, where white plastic cylinders (1.49 liter and 3.18 liter) coated on the inside with Stickem Special (Seabrite Enterprises, Emeryville, California) and Hara cone traps (Hara Products Ltd., Swift Current, Sask.) also were used. In the trap design experiment, traps were baited with 200 μg of a 4 : 1 blend of 3Z,6Z,9Z-20 : H with 3Z,6Z,9Z-19 : H. Dosages in other experiments are listed in the appropriate tables. All trials were set out in randomized block design, with four replications, unless otherwise stated.

Traps were baited with red rubber septa (Thomas Scientific, Philadephia, Pennsylvania) containing the synthetic lure blends. Lure blends were loaded onto septa as hexane solutions, followed by a few drops of a 10% solution of butylated hydroxytoluene in acetone as an antioxidant. Virgin females (1–5 days old) used as lures were placed in small screen cages inside sticky traps.

Traps were hung from metal posts 10–15 m apart within the dripline of shelter belt trees, at a height of approx. 1.5 m unless otherwise stated. Summed trap captures were transformed $[(X + 1)^{1/2}]$ and subjected to analysis of variance. The variances of the transformed data were tested for homogeneity with Bartlett's test. Significantly different means were separated by Duncan's (1955) multiple-range test. In tables, values followed by a common letter are not significantly different ($P > 0.05$).

Synthetic Chemicals. The syntheses of the biologically active 6Z,9Z-dienes,

3Z,6Z,9Z-trienes, and the corresponding *cis*-3,4-, *cis*-6,7-, and *cis*-9,10-epoxydienes described in this paper have been published elsewhere (Underhill et al., 1983; Wong et al., 1985; Millar et al., 1987). Chemicals used in field tests were >98% pure by gas chromatography.

All possible geometric isomers of the 3Z,6Z,9Z-trienes and of 6Z,9Z-19:H were synthesized by scrambling of the double bond geometries with aqueous nitrous acid (Sonnet, 1974). Thus, a mixture of 78 μl of 2 M aq. $NaNO_2$, 56 μl of 6 M HNO_3, and approx. 100 mg of 3Z,6Z,9Z–19:H in a 2-ml Reactivial (Pierce, Rockford, Illinois) was flushed with Ar, sealed, and heated for 1 hr at 75°C with vigourous stirring. The mixture was cooled and partitioned between sat. aq. $NaHCO_3$ and hexane. The hexane layer was washed with brine, dried over anhyd. Na_2SO_4, filtered through a 3-cm plug of silica gel in a pipete, and concentrated. Approx. 20 mg of the residue was fractionated by TLC [20 × 20-cm plate, 0.25-mm layer of 15% $AgNO_3$–silica (Analtech, Newark, Delaware), developed with 5% ether in hexane]. A portion of the plate was broken off and charred with H_2SO_4 to visualize the spots. Four fractions were cut, with R_f values of 0.59–0.70 (3*E*,6*E*,9*E*), 0.35–0.55 (*Z*,*E*,*E* isomers mixture), 0.13–0.35 (*Z*,*Z*,*E* isomers mixture), and 0.03–0.13 (residual 3Z,6Z,9Z isomer). Bands were scraped off the plates and the compounds were eluted from the scrapings with toluene (~10 ml). Isolated fractions were analyzed by capillary GC (DB-1701) under the identical conditions used in GC-EAD analyses of pheromone gland extracts.

The geometric isomers of 3Z,6Z,9Z–20:H were prepared in identical fashion. The geometric isomers of 6Z,9Z–19:H were prepared in a similar fashion, using a shorter reaction time (30 min).

RESULTS

In preliminary work, both male and female spring cankerworm adults were readily caught at Indian Head on sticky bands wrapped around tree trunks at a height of approximately 1 m. In 1984, five sticky bands caught a total of 5520 moths (4345 males and 1175 females) over a 28-day period from April 2 to 30. Significantly, large numbers of males were often caught on sticky bands that had not trapped any females, indicating that males were being caught as they emerged from the soil and climbed nearby vertical objects and were not simply being attracted by calling females stuck on the sticky bands. In a similar experiment run from April 2 to 30, 1986, five sticky bands caught 572 moths (491 males and 81 females). In both years, the beginning and the peak of male emergence as judged by captures on sticky bands appeared to occur a few days earlier than that of the female.

The identification of sex attractants and sex pheromone components for the

spring cankerworm was begun in 1983 with a number of male moths taken in black light traps. In electroantennogram (EAG) studies, antennae from male moths were challenged with 1-μg amounts of a variety of potential pheromone components (see list in Methods and Materials). From the large number of compounds tested, strong antennal responses were only seen to C_{18-22} 3Z,6Z,9Z-trienes, in particular to 3Z,6Z,9Z-19:H (~9 mV) and 3Z,6Z,9Z-20:H (~10 mV), with smaller responses (~1–4 mV) to mixtures of monoepoxides derived from nonselective oxidation of the trienes, and to C_{18-20} 6Z,9Z-dienes (~2–5 mV).

On the basis of the EAG data, a preliminary field test was conducted with lures consisting of 3Z,6Z,9Z-20:H (500 μg), alone or in combination (500:50 ratios) with its lower and higher homologs, 3Z,6Z,9Z-19:H and 3Z,6Z,9Z-21:H, respectively. Moths were caught in all traps, with lure blends composed of 3Z,6Z,9Z-20:H and 3Z,6Z,9Z-19:H being most attractive (data not shown). Captures were entirely species specific, with no other moth species being caught.

Further experiments in 1983 and 1984 were conducted to determine the most attractive blend of 3Z,6Z,9Z-19:H and 3Z,6Z,9Z-20:H (Table 1). The most attractive mixture appeared to be approximately a 1:4 blend of 3Z,6Z,9Z-19:H with 3Z,6Z,9Z-20:H, although the insects appeared to tolerate considerable variations in the blend ratio (from ~1:4 to ~1:20) with no loss of attraction. The optimal blends were as attractive as four caged females. One of

TABLE 1. CAPTURE OF SPRING CANKERWORM MOTHS IN TRAPS BAITED WITH MIXTURES OF 3Z,6Z,9Z-NONADECATRIENE AND 3Z,6Z,9Z-EICOSATRIENE OR VIRGIN FEMALES

Lure composition (μg)	Males captured[a]	
	1983	1984
3Z,6Z,9Z-19:H (500)	11^{d}	8^{f}
3Z,6Z,9Z-19:H (450) + 3Z,6Z,9Z-20:H (50)	33^{cd}	24^{e}
3Z,6Z,9Z-19:H (400) + 3Z,6Z,9Z-20:H (100)	43^{bc}	80^{d}
3Z,6Z,9Z-19:H (250) + 3Z,6Z,9Z-20:H (250)	77^{ab}	131^{c}
3Z,6Z,9Z-19:H (100) + 3Z,6Z,9Z-20:H (400)	111^{a}	224^{b}
3Z,6Z,9Z-19:H (50) + 3Z,6Z,9Z-20:H (450)	37^{cd}	291^{a}
3Z,6Z,9Z-19:H (25) + 3Z,6Z,9Z-20:H (475)		232^{b}
3Z,6Z,9Z-20:H (500)	20^{cd}	145^{c}
Females (4 per trap)	92^{a}	
Blank	0	2^{f}

[a] Traps were replicated three times. Pherocon-1CP (Zoecon Corp.) traps were used. In 1983, traps were set out from April 29 to May 9, at Indian Head, Sask.; in 1984, traps were set out from April 5 to 14, at Saskatoon, Sask. Traps were checked daily, and dead females were replaced. Values followed by the same letter are not significantly different ($P > 0.05$).

the components, 3Z,6Z,9Z-20:H, had considerable activity alone, while the other (3Z,6Z,9Z-19:H), when tested individually, gave trap captures similar to blank traps.

A dose–response experiment with a 1:4 blend of 3Z,6Z,9Z-19:H and 3Z,6Z,9Z-20:H on rubber septa revealed no significant difference in trap captures with a loading of 200–1000 μg (Table 2). Trap captures at the 50- and 100-μg levels were lower, but still significantly greater than blank traps.

In order to determine whether the attractants were actual pheromone components, a methylene chloride extract was prepared from ovipositor washes of several hundred field-collected females. The extract elicited strong EAG responses from male antennae.

A series of chemical tests were run on the extract. The active components did not appear to be esters or alcohols, since the EAG response was unchanged following either base hydrolysis or acetylation of the extract. Catalytic hydrogenation (H_2, 5% Pd on carbon) of the extract resulted in complete loss of EAG activity, indicating that the active components were unsaturated. On thin-layer chromatography with silica gel plates developed with hexane, EAG activity was concentrated in only one fraction, with R_f value ~0.4–0.6, indicating that the pheromone components were hydrocarbons, with no polar functional groups.

The extract was fractionated by micropreparative gas chromatography on a DB-5 capillary column, with fractions being collected in Dry Ice-cooled glass capillary tubes at 15-sec. intervals. Two major EAG-active fractions were noted, with retention times corresponding to 3Z,6Z,9Z-19:H and 3Z,6Z,9Z-20:H.

The extract was submitted to GC-MS analysis on a DB-5 column. In full-scan chemical ionization mode (CI; methane), a peak was found at the exact

TABLE 2. CAPTURE OF SPRING CANKERWORM MOTHS IN TRAPS BAITED WITH DIFFERENT DOSAGES OF 3Z,6Z,9Z-19:H + 3Z,6Z,9Z-20:H SEX ATTRACTANT BLEND (BLEND RATIO 1:4)

Lure dose (μg)	Males captured[a]
Blank	10[d]
50	96[c]
100	146[bc]
200	163[ab]
500	193[ab]
1000	222[a]

[a]Traps were replicated four times. Pherocon-1CP traps were used. Traps were set out from April 11 to 19, 1984, in Indian Head, Sask. Values followed by the same letter are not significantly different ($P > 0.05$).

retention time of 3Z,6Z,9Z-19:H, with strong ions at *m/z* 261 (48%, M−1), 263 (54%, M+1), and a weaker ion at *m/z* 291 (5%, M+29), for a molecular weight of *m/z* 262, corresponding to a molecular formula of $C_{19}H_{34}$, with three sites of unsaturation. A second peak was located at a retention time corresponding to that of 3Z,6Z,9Z-20:H, with strong ions at *m/z* 275 (27%, M−1), 277 (29%, M+1), and a weak ion at *m/z* 305 (2%, M+29), for a molecular weight of *m/z* 276, corresponding to a molecular formula of $C_{20}H_{36}$, with three sites of unsaturation. In both cases, the complete CI spectra closely matched those of synthetic 3Z,6Z,9Z-19:H and 3Z,6Z,9Z-20:H standards.

A portion of the extract (40 female equivalents) was spiked with standards (17:H and 21:H; 40 ng of each) and analyzed by full-scan electron impact GC-MS (70 eV). The EI mass spectra of the two components in the extract of the retention times of 3Z,6Z,9Z-19:H and 3Z,6Z,9Z-20:H matched the spectra of synthetic standards, and closely matched published mass spectra of 3Z,6Z,9Z-19:H (Becker et al., 1983; Wong et al., 1984) and 3Z,6Z,9Z-20:H (Underhill et al., 1983). In particular, diagnostic ions at *m/z* 55 $[CH_3CH_2CH{=}CH]^+$ and *m/z* 108 $[CH_3CH_2(CH{=}CH)_3H]^+$ in the spectra of both compounds located the first double bond at position 3, while the ions at *m/z* 206 $[C_9H_{19}(CH{=}CH)_3H]^+$ and *m/z* 220 $[C_{10}H_{21}(CH{=}CH)_3H]^+$ in the C_{19} and C_{20} compounds, respectively, located the position of the innermost double bond at C_9 (Underhill et al., 1983, and references therein; Wong et al., 1984). Both spectra also contained a series of ions at *m/z* 121, 135, etc., which are characteristic of polyunsaturated olefins (Underhill et al., 1983). In both cases, the relatively weak molecular ion was not seen; however, the molecular weights had been previously determined from the CI mass spectra.

At this point, the positions of the double bonds in the C_{19} and C_{20} trienes had been confirmed by the presence of diagnostic ions in the mass spectra. However, there was no hard analytical evidence that the double bonds were all *cis*. The nanogram amounts of insect-produced material were insufficient for NMR or GC-FTIR analysis. Consequently, all possible isomers of the 3,6,9-trienes were made by aqueous nitrous acid scrambling (Sonnet, 1974) of the double-bond geometries of 3Z,6Z,9Z-19:H and 3Z,6Z,9Z-20:H, to produce standards for GC retention time comparisons. The mixture of isomers of each chain length was readily separated by thin-layer chromatography on silica-$AgNO_3$ (15% $AgNO_3$) into fractions containing isomers with different numbers of *E* and *Z* double bonds (i.e., a 3*E*,6*E*,9*E* fraction, a combined fraction of the 3*E*,6*E*,9*Z*:3*E*,6*Z*,9*E*:3*Z*,6*E*,9*E* isomers, a combined fraction of the 3*E*,6*Z*,9*Z*:3*Z*,6*E*,9*Z*:3*Z*,6*Z*,9*E* isomers, and a fraction with the residual 3Z,6Z,9Z isomer). The fractions were spiked with heptadecane and tetracosane standards and compared to the pheromone gland extracts by GC analysis (DB-1701 column, 30 m × 0.32 mm ID, 25 μm film thickness). For both the C_{19} and C_{20} trienes, only the 3*E*,6*E*,9*E* isomer, and one of the isomers containing one *Z* and two *E* double bonds had retention times similar enough to those of

the 3Z,6Z,9Z isomer (retention time difference <0.1 min) to be potentially indistinguishable. On the basis of this analytical data, the possible isomeric configurations of the insect-produced trienes are limited to three of the possible eight isomers (3Z,6Z,9Z; 3*E*,6*E*,9*E*; an isomer containing one *Z* and two *E* double bonds).

Given the combined data from chemical tests, TLC R_f values, GC retention time matches, CI and EI mass spectra, comparisons with literature spectra, and field trapping data, we conclude that 3Z,6Z,9Z-19:H and 3Z,6Z,9Z-20:H are female-produced pheromone components for the spring cankerworm.

A coupled gas chromatography–electroantennogram (GC-EAD, DB-1701 column) run on the female extract, using a male antenna as a detector, revealed trace amounts of a third component eliciting a strong antennal response (Figure 1). This compound was tentatively identified as 6Z,9Z-19:H on the basis of a

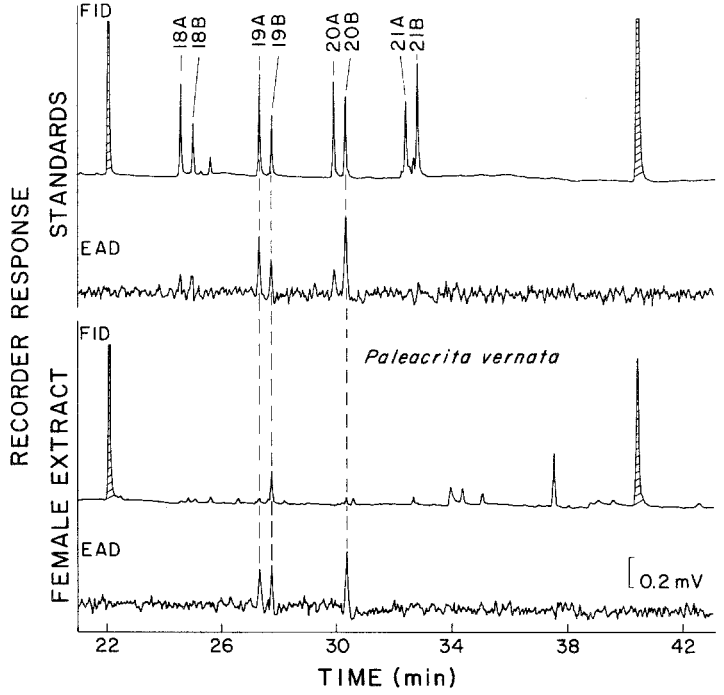


FIG. 1. Simultaneously recorded flame ionization detector (FID) and electroantennographic detector (EAD) traces using antennae of male *Paleacrita vernata*. Upper pair of traces are in response to a mixture of C_{18-22} 6Z,9Z-diene hydrocarbons (designated by the letter A and preceded by the carbon chain length) and 3Z,6Z,9Z-triene hydrocarbons (designated by the letter B). Internal standards (shaded peaks), 17:H and 24:H. Lower pair of traces are in response to conspecific female ovipositor extract. A 30-m DB-1701 column was used.

retention time match with an authentic standard. We presume that this compound had not been separated from 3Z,6Z,9Z-19:H in the previously mentioned preparative GC run, where 15-sec fractions had been taken.

The female ovipositor extract was reexamined by GC-MS with chemical ionization (methane). The retention time (Ultra-2 column) and the SIM profile of the unknown [400 pg standard: *m/z* 263 (100%, M−1), 249 (14%; M−15); unknown, *m/z* 263 (100%), 249 (15%)], matched those of an authentic 6Z,9Z-19:H standard.

Reexamination of the full-scan EI GC-MS data from the 40 female equivalent ovipositor extract revealed a small peak at the retention time of 6Z,9Z-19:H, from which an incomplete mass spectrum was extracted. The largest ion seen in this spectrum was at *m/z* 124, but the overall fragmentation pattern in the lower mass ranges closely matched that of the standard.

A sample of synthetic 6Z,9Z-19:H was submitted to the double-bond scrambling conditions (see above) to generate all possible geometric isomers. The scrambled mixture was separated into a fraction containing the 6*E*,9*E* isomer, a combined fraction containing the 6*E*,9*Z* and the 6*Z*,9*E* isomers, and a fraction containing residual 6Z,9Z-19:H. The 6*E*,9*E*, 6*Z*,9*E*, and 6*E*,9*Z* isomers all had retention times different by at least 0.1 min from that of 6Z,9Z-19:H.

Thus, the SIM GC-MS data, the incomplete full-scan EI data, and retention time matches on two different columns all support the tentative identification of the third GC-EAD active component as 6Z,9Z-19:H. However, the possibility that the insect-produced compound may be a positional isomer (e.g., 3Z,6Z-19:H) of 6Z,9Z-19:H cannot be completely eliminated.

Field trials in 1987 confirmed the importance of 6Z,9Z-19:H in the attractant blend (Table 3). Additions of 6Z,9Z-19:H to a basic bait comprised of 3Z,6Z,9Z-19:H and 3Z,6Z,9Z-20:H in a 1:4 ratio significantly increased trap captures. The 6Z,9Z-19:H compound was not attractive by itself. For reasons that are not known, the two-component blend performed poorly in this test (statistically no better than the blank traps).

A further study was carried out to evaluate the relative importance of each of the C_{19} components to the attractiveness of the blend. The two component blend of 3Z,6Z,9Z-20:H with 6Z,9Z-19:H (400:50 μg) proved to be as attractive as the full three-component blend (3Z,6Z,9Z-20:H/3Z,6Z,9Z-19:H/6Z,9Z-19:H, 400:100:50 μg) with 90 specimens attracted to each treatment, respectively, while the two-componment blend of 3Z,6Z,9Z-20:H with 3Z,6Z,9Z-19:H (400:100 μg) was significantly less attractive (29 specimens caught). The conclusions to be drawn from this are not obvious. 3Z,6Z,9Z-19:H synergizes the response to 3Z,6Z,9Z-20:H (Table 1), and elicits strong antennal responses in GC-EAD studies, but it apparently is not critical for optimal field attraction if 6Z,9Z-19:H is present in the blend.

TABLE 3. CAPTURE OF SPRING CANKERWORM MOTHS IN TRAPS BAITED WITH COMBINATIONS OF 3Z,6Z,9Z–19:H, 3Z,6Z,9Z–20:H, AND 6Z,9Z–19:H

Lure composition (μg)	Males captured[a]
3Z,6Z,9Z–19:H (100) + 3Z,6Z,9Z–20:H (400)	17[c]
3Z,6Z,9Z–19:H (100) + 3Z,6Z,9Z–20:H (400) + 6Z,9Z–19:H (10)	73[b]
3Z,6Z,9Z–19:H (100) + 3Z,6Z,9Z–20:H (400) + 6Z,9Z–19:H (50)	205[a]
6Z,9Z–19:H (500)	4[c]
Blank	5[c]

[a] Traps were replicated four times. Pherocon-1CP (Zoecon Corp.) traps were used. Traps were set out from April 6 to 9, 1987, in Saskatoon, Sask. Values followed by the same letter are not significantly different ($P > 0.05$).

Further studies were carried out to test for possible behavioral antagonists. In a brief field trial ($N = 3$) conducted at the end of the flight season in 1983, whereby 50 μg of the combined monoepoxides mixtures from nonselective oxidation of 3Z,6Z,9Z–19:H or 3Z,6Z,9Z–20:H were added to a basic bait comprised of 3Z,6Z,9Z–19:H and 3Z,6Z,9Z–20:H (1:4 ratio; 500 μg total weight), trap captures were reduced from 25 moths for the basic bait to one moth each for each of the epoxide-containing baits. These epoxides have been shown to be sex attractants and sex pheromone components for a number of sympatric lepidopteran species (Wong et al., 1985; Millar et al., 1990a–c).

A field experiment was conducted to determine which positional isomer of the C_{19} or C_{20} monoepoxydienes was the behavioral antagonist. The attractive three-component blend (6Z,9Z–19:H, 3Z,6Z,9Z–19:H, 3Z,6Z,9Z–20:H, 1:2:8) was spiked with 10 μg of each of the C_{19} or C_{20} epoxides. At this dosage only 6Z,9Z,*cis*-3,4-epoxy-19:H caused significant reduction in trap captures (Table 4).

Two further trials were carried out to test the effects of trap design and trap height. In the first experiment, four different trap types were tested for trapping efficiency (April 12–26, 1984). Total trap catches in small and large cylinders (221 and 167 moths, respectively) were significantly greater than in Pherocon 1CP traps (61 moths). The Hara traps were unsatisfactory, catching only six moths.

The second experiment, to test the effects of trap height, was conducted by hanging four attractant-baited sticky traps at different heights in each of five trees. Trap captures were significantly correlated with trap height, with trap captures increasing approximately fourfold in going from a trap height of 2 m to a height of 8 m (Table 5).

TABLE 4. EFFECT OF RACEMIC MONOEPOXIDES DERIVED FROM 3Z,6Z,9Z-19:H OR 3Z,6Z,9Z-20:H ON ATTRACTIVENESS OF BASIC LURE (3Z,6Z,9Z-19:H + 3Z,6Z,9Z-20:H + 6Z,9Z-19:H, 100:400:50 μg, RESPECTIVELY) FOR SPRING CANKERWORM MOTHS

Lure composition (μg)	Males captured[a]
Basic bait	194[ab]
Basic bait + 6Z,9Z-*cis*-3,4-epoxy-nonadecadiene (10 μg)	77[c]
Basic bait + 3Z,9Z-*cis*-6,7-epoxy-nonadecadiene (10 μg)	306[a]
Basic bait + 3Z,6Z-*cis*-9,10-epoxy-nonadecadiene (10 μg)	147[abc]
Basic bait + 6Z,9Z-*cis*-3,4-epoxy-eicosadiene (10 μg)	100[bc]
Basic bait + 3Z,9Z-*cis*-6,7-epoxy-eicosadiene (10 μg)	249[a]
Basic bait + 3Z,6Z-*cis*-9,10-epoxy-eicosadiene (10 μg)	329[a]

[a]Traps were replicated four times. Pherocon-1CP (Zoecon Corp.) traps were used. Traps were set out from April 14 to 16, 1987, in Indian Head, Sask. Traps were placed along a Siberian elm shelter belt, in one continuous north-south line, with guard traps (same basic bait as above) at each end of the line. Values followed by the same letter are not significantly different ($P > 0.05$).

TABLE 5. CAPTURE OF SPRING CANKERWORM MOTHS IN PHEROMONE-BAITED TRAPS HUNG AT DIFFERENT HEIGHTS

Trap height (m)	Males captured[a]
2	113[c]
4	152[c]
6	253[b]
8	429[a]

[a]Pherocon-1CP (Zoecon Corp.) traps baited with a 4:1 mixture of 3Z,6Z,9Z-20:H/3Z,6Z,9Z-19:H (200 μg total) on rubber septa were used. A complete replication (all four heights) was placed in each of five Siberian elm trees at Indian Head, Sask. Traps were counted at 3 to 4-day intervals from April 16 to 30, 1984. Values followed by the same letter are not significantly different ($P > 0.05$).

DISCUSSION

Large numbers of moths of both sexes were caught on unbaited sticky bands wrapped around tree trunks. It was expected that the wingless females, upon eclosion, would move to nearby vertical objects and climb them to begin calling. Male moths apparently exhibit similar behavior after eclosion while their wings are drying and expanding fully, despite the potentially greater risk of predation on the exposed tree trunk.

The sex ratio of adults appeared to be strongly biased in favor of males, based on sticky band captures. Although these band captures may not accurately measure sex ratios due to differences in male and female behaviors, only 81 of the 572 moths caught in 1986 were females. Of the 491 males caught, 173 were caught on sticky bands on which no females had been trapped. Even discounting the probable increased catches of males on sticky bands with trapped females, the sex ratio still strongly favored males. This skewed sex ratio had been previously observed by Packard (1876), where inked bands wrapped around tree trunks caught approximately five times as many males as females. Similarly, Young (1918) reported a 3 : 1 ratio of males versus females caught in an emergence trap placed on the ground.

It has been observed that male moths of another geometrid species, the fall cankerworm, *Alsophila pometaria* Harris, orient to vertical silhouettes. Upon landing on a vertical surface such as a tree trunk, the male moths then actively search for females by running up and down the tree surface (Palaniswamy et al., 1986). If spring cankerworm males also use this type of active searching behavior, a disproportionately large number of male moths could be caught on the sticky bands.

The analytical and field trapping data suggest that the female-produced sex pheromone of the spring cankerworm may consist of a blend of at least three components, two of which (3Z,6Z,9Z-19 : H and 3Z,6Z,9Z-20 : H) have been identified from female pheromone glands, and with a third component tentatively identified as 6Z,9Z-19 : H. The lure ratio was 1 : 2 : 8 of diene-C_{19} triene-C_{20} triene.

On the basis of analytical chemistry data alone, three of the eight possible triene double-bond configurations could not be distinguished (3Z,6Z,9Z; 3*E*,6*E*,9*E*; one of the *Z*,*E*,*E* isomers). The bioassay data (Tables 1–4) demonstrated that male moths were strongly attracted to blends of 3Z,6Z,9Z-19 : H and 3Z,6Z,9Z-20 : H. The possibility that the insects would be attracted to blends of these trienes when the actual pheromone components were isomers with two or three *E* double bonds seems remote, because of the large differences in molecular shape between these isomers and the 3Z,6Z,9Z-trienes. In other words, the bioassay data strongly suggest that the C_{19} and C_{20} 3Z,6Z,9Z-trienes are the only reasonable isomeric structures for the pheromone components, from the three possible choices identified from GC and GC-MS data. In addition, to our knowledge there are no known instances where an insect is strongly attracted to geometric isomers of its sex attractant or pheromone component.

There have been a number of other reports of unsaturated hydrocarbons such as these as lepidopteran sex attractants and pheromones. In particular, 3Z,6Z,9Z-19 : H has been reported as a sex attractant or pheromone component for 16 geometrid and one noctuid species (Becker et al., 1987; Bögenschutz et al., 1985; Buser et al., 1985; Millar et al., 1990a–c; Szöcs et al., 1984, 1987;

Wong et al., 1984, 1985), while 3Z,6Z,9Z-20:H has been reported as an attractant or pheromone component for three geometrid (Wong et al., 1985) and five noctuid species (Heath et al., 1983; Landolt et al., 1986; McLaughlin and Heath, 1989; Underhill et al., 1983; Wong et al., 1985). There have been only three previous reports of 6Z,9Z-19:H in attractant blends, all for geometrid moth species (Bögenschutz et al., 1985; McDonough et al., 1986; Szöcs et al., 1984). Most of these attractants and pheromones are behaviorally active as blends of several components, as was found for the spring cankerworm, with the components varying in either chain length or the degree of unsaturation. For example, the fall cankerworm *Alsophila pometaria* Harris, which is sympatric with the spring cankerworm and has a similar host range, utilizes a blend of 3Z,6Z,9Z-19:H, 3Z,6Z,9Z,11Z-19:H, and 3Z,6Z,9Z,11E-19:H in its pheromone (Wong et al., 1984). Alternatively, the noctuid species *Anticarsia gemmetalis* Hübner (Heath et al., 1983), *Mocis disseverans* Walker (Landolt et al., 1986), and *Caenergina erechtea* Cramer (Underhill et al., 1983) all have pheromones containing 3Z,6Z,9Z-20:H in combination with 3Z,6Z,9Z-21:H.

There are also numerous examples of pheromone blends consisting of one or more unsaturated hydrocarbons in combination with unsaturated epoxides (review to 1986, Arn et al., 1986; Millar et al., 1987; 1990a-c), aldehydes (Hill and Roelofs, 1981; Hill et al., 1982), and ketones (Buser et al., 1985).

At this point, the interactions between the three pheromone components are not clear. The two-component blend of 6Z,9Z-19:H with 3Z,6Z,9Z-20:H is as attractive as the full three-component blend, while the two component blend of 3Z,6Z,9Z-19:H and 3Z,6Z,9Z-20:H is significantly less attractive. This suggests that 3Z,6Z,9Z-19:H may be a redundant component, at least in terms of the end result of catching moths in traps. The phenomenon of redundancy, and in some cases interchangeable components, has been studied in depth and comprehensively documented for the cabbage looper moth (Linn and Roelofs, 1989).

However, it must be emphasized that 3Z,6Z,9Z-19:H may possibly be redundant only in terms of the end result of catching moths in the traps. This end result may not take into account the numerous behavioral steps involved in orientation, upwind flight, and contacting an attractant source, as well as the subsequent pheromone-mediated events of courtship and copulation. It has been demonstrated in a number of lepidopteran species that each of the steps in this behavioral hierarchy is affected by minor components in a pheromone or attractant blend (e.g., Linn and Roelofs, 1989; Palaniswamy et al., 1983; Wong et al., 1984). The fact that the male antennae respond strongly to 3Z,6Z,9Z-19:H, and that 3Z,6Z,9Z-19:H synergizes the response to 3Z,6Z,9Z-20:H suggests a role for this compound, but its exact function is not clear.

The experiment testing trap design showed that sticky traps were more

effective traps for male moths than the Hara cone traps. Field observations of spring cankerworm moths placed in the cone traps demonstrated that the moths could escape quite easily through the relatively large mesh on the ends of the traps. However, it has been shown that the effectiveness of cone traps can be increased considerably by placing an insecticidal strip inside the trap to knock down and kill moths that enter the trap (e.g., Mitchell and Heath, 1986; Underhill et al., 1987). As these traps have the considerable advantages of being reusable and less messy than sticky traps, further field trials with cone traps containing insecticidal strips may be warranted.

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ANALYSIS AND FIELD EVALUATION OF VOLATILE BLEND EMITTED BY CALLING VIRGIN FEMALES OF BEET ARMYWORM MOTH, *Spodoptera exigua* (HÜBNER)

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Abstract—The volatile blend emitted by calling virgin beet armyworm females was collected and analyzed by capillary gas chromatography and mass spectrometry. Five compounds were detected and identified in this blend: (*Z*)-9-tetradecenyl acetate, (*Z*)-9-tetradecen-1-ol, (*Z,E*)-9,12-tetradecadienyl acetate (*Z,Z*)-9,12-tetradecadienyl acetate, and (*Z*)-11-hexadecenyl acetate. They were emitted in a ratio of 47.9:4.0:40.2:6.5:1.7, respectively. This five-component blend and several other blends with one or more components deleted were formulated on rubber septa to release the components in about the same ratios as the females. Field tests of these formulations in bucket traps indicated that a blend of (*Z*)-9 tetradecen-1-ol, (*Z,E*)-9,12-tetradecadienyl acetate, and (*Z*)-11-hexadecenyl acetate was most effective for trapping beet armyworm males and that (*Z*)-9-tetradecenyl acetate actually decreased trap captures. Blends that did not contain both (*Z*)-9-tetradecen-1-ol and (*Z,E*)-9,12-tetradecadienyl acetate were ineffective as trap baits.

Key Words—Trapping, volatile blend, sex pheromone, pheromone blends, trap bait, capture, beet armyworm moth, *Spodoptera exigua*, Lepidoptera, Noctuidae.

INTRODUCTION

The beet armyworm (BAW), *Spodoptera exigua* (Hübner), is a pest of a wide variety of crops in many areas of the world and is one of the three most impor-

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tant pest species in the genus *Spodoptera* in North America. There have been several investigations of its sex pheromone in the last two decades. Brady and Ganyard (1972) identified (*Z,E*)-9,12-tetradecadien-1-ol acetate (*Z9,E*12-14:AC)[3] from abdominal tips of BAW females. However, *Z9,E*12-14:AC is ineffective as a trap bait for BAW males in the field (Mitchell and Doolittle, 1976). Subsequently, we isolated and identified a series of 14-carbon alcohols and acetates from hexane washes of virgin BAW females collected 6–7 hr into the scotophase (Tumlison et al., 1981). Not all of the compounds identified were required to elicit a response by males in laboratory assays or to capture males in sticky traps in the field. A blend of only two, *Z9,E*12-14:AC and Z9-14:OH, was equal to virgin females in effecting capture of males in traps. The other compounds found in the washes of females were S-14:OH, *E*9-14:OH, *Z9,E*12-14:OH, *Z9,Z*12-14:OH, S-14:AC, *Z*7-14:AC, *Z*9-14:AC, *E*9-14:AC, and *Z9,Z*12-14:AC. Later, we established that a blend of *Z9,E*12-14:AC (100 μg) and Z9-14:OH (10 μg) on rubber septa produced consistently higher captures of BAW males in sticky traps than did virgin females (Mitchell et al., 1983). None of the other nine components identified from female washes enhanced trap captures when admixed with the binary attractant blend. Omission of Z9-14:OH from the blend significantly reduced trap captures.

Two other research groups have confirmed the effectiveness of the two-component blend as a trap bait for BAW males. Cheng et al. (1985) in Taiwan found that a blend of *Z9,E*12-14:AC and Z9-14:OH in a 10:1 ratio formulated in an oil carrier containing antioxidant and dispensed from micro nylon tubes was effective for the capture of BAW males in sticky and water traps. Similarly, Trumble and Baker (1984) demonstrated that a wing trap baited with a 100:10 μg blend of these same compounds formulated in polyethylene caps or hollow fibers was effective in capturing BAW males in California.

In contrast, Persoons et al. (1981) identified five acetates, S-14:AC, Z9-14:AC, Z11-14:AC, *Z9,E*12-14:AC, and *Z9,Z*12-14:AC, in hexane rinses of pheromone glands excised from BAW female abdomens. They did not find any alcohols and reported that trap captures of BAW males in sticky traps baited with polyethylene caps containing a blend of *Z9,E*12-14:AC (0.6 mg) and Z9-14:AC (0.3 mg) were equivalent to those baited with virgin BAW females. They also reported Z9-14:OH not to be an essential component of the BAW pheromone.

Rogers and Underhill (1981) reported significant captures of BAW males in sticky traps baited with rubber septa containing 0.1 mg *Z9,E*12-14:OH and

[3] A standard shorthand notation for pheromone molecules of this type will be used in this paper. For example (*Z*)-9-tetradecen-1-ol is abbreviated Z9-14:OH; the corresponding acetate is Z9-14:AC; tetradecan-1-ol is abbreviated S-14:OH.

0.01 mg *Z*9, *E*12–14 : AC. The lack of a BAW colony precluded these workers from determining the performance of these baits relative to virgin females.

Because of the conflicting reports in the literature and the worldwide importance of the BAW as a crop pest, we decided to reinvestigate the pheromone of this species to determine as accurately as possible the composition of the pheromone emitted by BAW females. We report here the collection and analysis of volatiles emitted by calling BAW families, the formulation of synthetic blends on rubber septa to release the components in the same ratios that they are released by females, and the evaluation of these formulations in traps in the field.

METHODS AND MATERIALS

Rearing. BAW moths used in this study were reared in the laboratory on artificial diet (Burton, 1970). After sexing, female pupae were placed in 35 × 35 × 35-cm Plexiglas cages and maintained under a 14 : 10 hr reversed light–dark cycle at 26°C and 65% relative humidity. Daily transfers of unenclosed pupae to empty cages ensured that each cage contained adult females of the same age. Cotton balls saturated with an 8% sugar water solution were placed in each cage to feed the adults.

Pheromone Extraction and Collection. Pheromone glands were excised from actively calling 2- to 4-day old females collected between 6 and 9 hr after the beginning of the dark period. Four to eight glands were immersed for about 30 sec in 20 μl of hexane (Fisher Scientific Co., 99 mol% pure) in a microvial. Then the glands were removed, and the solution was allowed to evaporate to about 2 μl and analyzed directly by capillary GC. After initial analyses to locate peaks in the chromatogram, 2 ng of a noninterfering internal standard, S–16 : AC, in 2 μl of hexane was added to the extracts before evaporation and analysis.

The apparatus used to collect pheromone emitted by calling BAW females was similar to the one used to collect volatiles from fall armyworm females (Tumlinson et al., 1986). Compressed air was passed through a charcoal prefilter at 1 liter/min and the airstream was then split sending 0.7 liter/min through a gas dispersion chamber containing distilled water. The two airstreams were then recombined and passed through a coarse glass frit to provide a laminar flow of humidified air through a glass aeration chamber (50 cm × 20 cm ID). Entrained volatiles were collected on a small charcoal filter, similar to the one described by Grob and Zürcher (1976), through which the air exited the chamber.

Active 2- to 4-day-old females were selected from the cages just prior to the period of maximum calling activity, approximately 6 hr after the beginning

of the dark period (Tingle and Mitchell, 1975). Approximately 10 females were placed in the aeration chamber, and volatiles entrained in the air passing over them were collected for 1–2 hr. Then the collection filter was removed and rinsed with three 20-μl portions of methylene chloride (B&J GC^2, American Burdick and Jackson, Muskegon, Michigan) followed by 20 μl of hexane (B&J GC^2). The rinse solutions were combined and concentrated to about 5 μl by gentle warming. A system blank collection was made on the same day in the same manner by using a clean chamber without insects. Again, after preliminary analyses, 10 ng of S–16:AC in 10 μl of hexane was added as an internal standard prior to concentration of the filter rinses. Quantitation of the components of the volatile blend was by comparison of the peak areas with that of the internal standard.

Chemical Analyses. GC analyses were conducted on a Varian model 3700 GC and a Hewlett-Packard model 5890 GC, both equipped with splitless capillary injector systems and flame ionization detectors. A Perkin Elmer chromatographic data system was used for data collection, storage, and subsequent analysis. Helium was used as the carrier gas at a linear flow velocity of 19 cm/sec. Columns used for all analyses were: OV-101, 50 m × 0.25 mm ID fused silica, 0.25 μm film thickness, initial temperature 60°C or 1 min, then temperature programmed (TP) at 10°C/min to 200°C; CPS-1 (bonded cyanopropyl methyl silicone, Quadrex Corp., New Haven, Connecticut) 50 m × 0.25 mm ID fused silica, 0.25 μm film thickness, initial temperature 60°C, TP at 5°C/min to 180°C. All injections were made in the splitless mode with a 30-sec delay before injector purging. Injector and detector temperatures were 240°C and 260°C, respectively.

Samples also were analyzed by GC-mass spectrometry (MS) with a Nermag model R1010 mass spectrometer in the chemical ionization mode with methane as the reagent gas. The OV-101 capillary column described above was used for GC-MS analyses with He carrier gas. Spectra of natural products were compared with those of authentic synthetic standards.

All synthetic compounds used in this study were obtained from commercial sources and were analyzed on both capillary GC columns to determine their purity. Compounds that were less than 98% pure were purified by HPLC on a $AgNO_3$-coated silica column eluted with toluene (Heath et al., 1977) and reanalyzed by GC to ensure that they were greater than 99% pure.

Formulations. Synthetic blends were formulated on 5 × 9-mm rubber septa (A. H. Thomas Co.) for all biological tests. Septa were Soxhlet extracted with methylene chloride for 24 hr and air dried prior to loading. The percentage of a component in a blend to be loaded onto septa was calculated on the basis of its relative volatility, determined from retention indices on liquid crystal capillary GC columns (Heath and Tumlinson, 1986), by a method developed to predict release ratios of components of a blend from rubber septa (Heath et al.,

1986). All blends prepared for formulation on septa were analyzed by capillary GC to confirm the component ratios. Each septum was loaded with 100 μl of a hexane solution of the blend pipetted into the well on the large end of the septum. Septa were aired for two days at room temperature before use.

To determine the release rate of the components of the various blends and to verify that the release ratios were similar to the calculated values, septa were aerated in an apparatus similar to the one used to collect volatiles from females. A septum was placed in a 1.26-cm (ID) $\times$ 2.4-cm aeration chamber and made from Swagelok (Crawford Fitting Co., Solon, Ohio) brass fittings. The downstream end of the chamber consisted of a reducer with a 5/8-in. (OD) tube coupled to a 1/4-in. (ID) threaded male fitting to which the charcoal filter trap was connected via a 1/4-in. (OD) brass tube with a Teflon sleeve. The 5/8-in. tube was connected to a 5/8- to 3/8-in. reducing union through which the air entered the aeration chamber. An airflow of 1 liter/min was maintained over the septum for 2–3 hr, the volatiles trapped on the charcoal filter were eluted as previously described, an internal standard was added, and the solution was concentrated and analyzed by capillary GC on the CPS-1 column.

Field Tests. Trapping experiments were conducted in Alachua County, Florida, during summer 1988 and spring 1989. Tests were set out in randomized complete blocks adjacent to corn and sorghum fields in areas where *Amaranthus* spp., a favored weed host of the beet armyworm, was abundant. International Pheromones Moth Traps (bucket traps) mounted ca. 1 m above the ground on metal poles were positioned ca. 60 m apart within blocks (lines); each of the three blocks was oriented perpendicular to the prevailing wind. The distance between blocks was ca. 150 m.

The traps were baited with individual rubber septum dispensers. A small piece of Vapona insecticide strip was placed inside the buckets as a killing agent for trapped moths. The number of moths captured per trap was recorded every two to four days, and each recorded collection was considered a replicate. The treatments (lure and trap) were rerandomized after each collection. For statistical analyses, the number of moths captured per trap was converted to $\sqrt{x + 0.1}$ and subjected to an analysis of variance. Differences between means were separated by using Duncan's (1955) multiple-range test (Scholtzhauer and Littel, 1987). Regression analysis (Steel and Torrie, 1960) was used to evaluate the response of the beet armyworms to different pheromone doses.

RESULTS AND DISCUSSION

Initially, the pheromone glands from calling females were excised, rinsed with hexane, and the hexane solution analyzed to identify the compounds that were present, although previous experience and literature reports indicate that

the components and ratios present in the gland may differ from those released by calling females (Tumlinson, 1988). Analysis on both capillary columns indicated that there were six peaks consistently present above the background peaks of impurities in the solvent. These peaks coincided in retention times on both columns with Z9-14:OH, Z9,E12-14:OH, Z9-14:AC, Z9,E12-14:AC, Z9,Z12-14:AC, and Z11-16:AC. A peak coincident in retention time with Z7-14:AC and about 1% as large as the Z9-14:AC peak was occasionally present, but it was not present consistently in quantities large enough to distinguish it from background impurities. Also not present in quantities detectable above background impurities were S-14:OH, E9-14:OH, Z9,Z12-14:OH, S-14:AC, and E9-14:AC, which we found previously in solvent washes of females (Tumlinson et al., 1981). There also was no peak on either column at the correct retention time for Z11-14:AC, reported to be present in gland extracts by Persoons et al. (1981). GC-MS analysis confirmed that the six peaks detectable by GC had spectra consistent with the structures assigned on the basis of retention times and did not detect any of the other previously identified compounds.

Only five peaks were detected above system blank background impurities by GC analysis on both capillary columns of the volatiles collected from calling BAW females. These peaks coincided in retention times on both columns with five of the compounds found in the gland rinse. Only the Z9,E12-14:OH was absent. The ratios and rates at which they were released by the females are listed in Table 1. GC-MS analysis confirmed the identification of these compounds.

A blend of the five compounds identified in the volatiles released by calling females was formulated on rubber septa. Based on the relative vapor pressures of the compounds (Heath and Tumlinson, 1986), we calculated the required percent of each component in the blend loaded on the septa to give a release

TABLE 1. VOLATILE COMPONENTS OF PHEROMONE BLEND RELEASED BY CALLING BAW FEMALES

Compound	Release rate[a]		Release ratio[a]	
	ng/FH[b]	(±SD)	%	(±SD)
Z9-14:AC	1.02	(±0.22)	47.9	(±2.2)
Z9-14:OH	0.09	(±0.04)	4.0	(±1.2)
Z9,E12-14:AC	0.86	(±0.19)	40.2	(±3.1)
Z9,Z12-14:AC	0.15	(±0.09)	6.5	(±2.7)
Z11-16:AC	0.03	(±0.01)	1.7	(±7.0)

[a] Means of six replicates. Volatiles were collected from 5 to 10 females for each replicate.
[b] FH = female hour.

ratio that would be the same as that from the females (Heath et al., 1986). Volatiles were collected from the septa in the laboratory and analyzed to determine the actual release ratio and rate of the components under laboratory conditions. Several other blends in which one or more of the components were deleted from the natural blend were formulated in the same manner and the volatiles released were analyzed. Each septum was loaded with 200 μg of the total blend. The percent loads and measured release ratios of the components of each blend formulated on septa are given in Table 2. Half the septa loaded

TABLE 2. SYNTHETIC BLENDS FORMULATED ON RUBBER SEPTA FOR FIELD TESTS AND RELEASE RATIOS (PERCENT) OF COMPONENTS OF EACH BLEND AS MEASURED IN THE LABORATORY (N = 8 FOR EACH BLEND)

Blend	Components	Z9-14:AC	Z9-14:OH	Z9,E12-14:AC	Z9,Z12-14:AC	Z11-16:AC
B5-4	Load (%)[a]	40.9	1.5	45.9	6.2	5.5
	Mean release ratio	49.2	3.0	40.4	5.6	1.9
	(±SD)	(±1.9)	(±0.6)	(±1.5)	(±0.5)	(±0.5)
B4-5	Load (%)[a]	0	2.4	77.3	11.1	9.2
	Mean release ratio	0	7.0	78.5	11.4	3.3
	(±SD)	—	(±1.3)	(±2.1)	(±1.0)	(±0.9)
B4-6	Load (%)[a]	43.7	1.5	49.5	0	5.3
	Mean release ratio	51.3	3.6	43.0	0	2.1
	(±SD)	(±2.2)	(±0.9)	(±1.7)		(±0.5)
B4-7	Load (%)[a]	42.8	1.4	49.1	6.8	0
	Mean release ratio	48.9	3.6	41.4	6.2	0
	(±SD)	(±2.0)	(±1.2)	(±1.6)	(±0.5)	
B4-8	Load (%)[a]	42.5	0	46.3	6.1	5.1
	Release ratio not measured					
B4-9	Load (%)[a]	75.3	2.6	0	12.2	9.9
	Release ratio not measured					
B3-2	Load (%)[a]	0	2.5	87.2	0	10.3
	Mean release ratio	0	6.8	90.0	0	3.3
	(±SD)		(±0.7)	(±1.4)		(±1.2)
B2-2	Load (%)[a]	0	2.8	97.2	0	0
	Mean release ratio	0	6.8	93.2	0	0
	(±SD)		(±0.6)	(±0.6)		
B2-1	Load (%)[b]	0	9.0	91.0	0	0
	Mean release ratio	0	19.9	80.1	0	0
	(±SD)		(±3.8)	(±3.8)		

[a] Each septum was loaded with 200 μg of the total blend.
[b] Blend B2-1 was loaded at a dose of either 110 μg/septum or 200 μg/septum.

with each blend were used in traps in the field tests (see later) then returned to the lab, and the release ratios and rates measured. There were no significant differences in release ratios between septa aged in the field for one to three weeks and those held in glass vials at room temperature in the laboratory for a few days before volatiles were collected. Although the quantity of *Z*9,*E*12–14 : AC loaded per septum varied from 91.7 ng for blend B5-4 to 194.4 ng for blend B2-2, the release rate of this compound was fairly consistent. From laboratory septa, it ranged from a mean of 7.75 ng/hr (±1.24 SD) for blend B5-4 to 9.42 ng/hr (±2.49 SD) for blend B2-2, while from field aged septa, it ranged from 5.69 ng/hr (± 0.53 SD) for blend B5-4 to 8.02 ng/hr (±3.59 SD) for blend B2-2. The mean rate of release of *Z*9,*E*12–14 : AC from all laboratory septa loaded with 200 μg of the total blend was 8.55 ng/hr (±2.1 SD, N = 24), while the septa that had been aged in the field (measured for all septa loaded with 200 μg total blend) released this compound at a mean rate of 6.61 ng/hr (±2.0 SD, n = 24).

Preliminary field tests using the wind-oriented trapping (WORT) system (Mitchell et al., 1988) for direct comparison of two blends showed that there was no difference in captures of BAW males in sticky traps baited with 200 μg of the five-component blend or a blend of *Z*9,*E*12–14 : AC/*Z*9–14 : OH (100 : 10 μg) on rubber septa. However, when the same two blends were compared in bucket traps using the WORT system, the five-component blend captured significantly more males (X = 4.9/trap/night) than the two-component blend (X = 2.4/trap/night). Therefore the bucket traps were used in all subsequent tests.

In field tests 1–5 (Table 3), the effectiveness of the blend of five components released by females was compared with several blends in which one or more components were deleted. The blends, formulated as indicated in Table 2, were designed to release the components in the same ratio that they were released by calling females (Table 1). Also included in the test was the 10 : 1 blend of *Z*9,*E*12–14 : AC and *Z*9–14 : OH (Mitchell et al., 1983) at two doses (100 μg of *Z*9,*E*12–14 : AC and 200 μg of the blend). These tests were designed to determine if all five compounds released by females are necessary to trap males. Interestingly, the data (Table 3) indicate that elimination of *Z*9–14 : AC from the five-component blend (test 1) and from the B4-6 four-component blend (test 3) results in an increase in the capture of BAW males in the bucket traps. Also, B4-5 appears to be more attractive than B5-4 in test 2, although the data are not significantly different. The data also indicates that the three-component blend was equal or superior to all other blends in all tests (1, 3, and 5) in which it was compared. Thus it appears that *Z*9,*E*12–14 : AC, *Z*9–14 : OH, and *Z*11–16 : AC are required for optimum captures of BAW males with this type of trap and that *Z*9,*Z*12–14 : AC has no effect, while *Z*9–14 : AC, the major component in the blend released by females, may actually reduce captures when it is incorporated in the blend.

TABLE 3. CAPTURE OF MALE BEET ARMYWORMS IN BUCKET TRAPS BAITED WITH DIFFERENT PHEROMONE BLENDS ON RUBBER SEPTA DISPENSERS. ALACHUA COUNTY, FLORIDA, 1988

	Mean number (±SE) moths captured[a]							
Reps	B5-4	B4-5	B4-6	B4-7	B3-2	B2-2	B2-1[b]	Control
Test 1 (July 5–August 1)								
22	11.7 ± 1.4b	18.0 ± 2.6a	9.1 ± 1.6bc	9.1 ± 2.0bc	18.4 ± 2.5a	11.2 ± 2.0bc	8.3 ± 1.4c	Od
Test 2 (August 8–23)								
11	63.6 ± 15.5a	81.7 ± 19.3a				27.3 ± 8.6b		Oc
Test 3 (August 8–23)								
11	28.9 ± 6.7b		18.2 ± 4.6b		66.8 ± 16.1a			Oc
Test 4 (August 8–23)								
11	39.8 ± 13.4a			38.9 ± 12.2a			58.3 ± 20.2a	Ob
Test 5 (August 15–29)								
10	75.7 ± 21.5b				132.5 ± 36.0a	71.3 ± 21.9b		Oc

[a] Means within tests with different letters are significantly different, $P < 0.05$, Duncan's multiple-range test (Duncan, 1955).
[b] All septa were loaded with 200 μg of their respective blends except those for B2-1 in test 1 which were loaded with a mixture of 100 μg Z9,E12-14:AC and 10 μg Z9-14:OH. The composition of each blend and release ratios are given in Table 2.

Since test 1 indicated that blend B2-1 at a dose of 100 μg of Z9,E12-14:AC was less attractive than the five-component blend, while B2-1 at a dose of 200 μg of the total blend was at least as attractive as B5-4 in test 4, we compared both doses of B2-1 with the five-component and three-component blends in the same test (test 6, Table 4). The results indicated that captures with B2-1 at 200 μg were not significantly higher than with B2-1 at the lower dose or with B5-4. Since Persoons et al. (1981) reported that Z9-14:OH was not an essential component of the BAW pheromone, we compared a blend of all the acetates found in volatiles released by females with the five-component and three-component blends (test 7, Table 4). We also compared a blend in which only Z9,E12-14:AC was eliminated (test 8, Table 4). The results of these latter two tests confirmed our earlier findings that both Z9-14:OH and Z9,E12-14:AC are essential components of the BAW pheromone.

The release rate of compounds or blends from rubber septa is dependent on wind speed and temperature, two highly variable parameters in the field. The release rate of the pheromone blend by calling females is probably less dependent on these parameters. Thus, although the release rate of Z9,E12-14:AC from rubber septa loaded with 200 μg of the total blend was about 8- to 10-fold that from females in the laboratory, the relative release rates in the field could be quite different. Therefore, we compared a range of doses of the three-component blend on rubber septa with four virgin females in bucket traps in the field. The results (Figure 1) suggest that a 100-μg dose of this blend on a septum would be approximately equal to four females under the conditions of our test. We did not measure the release rates of the components from a septum loaded with a 100-μg dose. However, previous studies with compounds of this type (unpublished) indicate that halving the dose in this dose range reduces the release rate by about 25%. Thus, under our laboratory conditions a septum loaded with 100 μg of the pheromone blend should release it at approximately the same rate as six or seven females combined.

Although our data indicate that Z9-14:AC reduces trap captures, it is difficult to understand why a compound released by females of a species as a major component of the volatile blend that contains their pheromone would reduce the attraction of conspecific males to the females. It seems more likely that this compound plays some as yet undefined role in mating communication in this species. It is possible that the reduction in trap captures could be an artifact of our experimental design. The release ratios from the rubber septa were, within experimental error, the same as the ratio released by females. The release rates from the septa were significantly higher than the release rate from a female, and it is possible that a comparison of the different blends at a lower dose would have yielded different results. However, the data in Figure 1 do not indicate that 200 μg was an excessively high dose in these tests. Further tests are planned to evaluate several blends at lower doses and also different trap designs.

TABLE 4. CAPTURE OF MALE BEET ARMYWORM MOTHS IN BUCKET TRAPS BAITED WITH DIFFERENT PHEROMONE BLENDS ON RUBBER SEPTA DISPENSERS (ALACHUA COUNTY, FLORIDA, 1989)

	Mean number (±SE) moths captured[a]							
Reps	B5-4	B4-8	B4-9	B3-2	B2-1	B2-1[b] (110 μg)	Females (4)	Control
Test 6 (April 4–May 5)								
21	7.0 ± 0.9ab			7.9 ± 1.1a	5.4 ± 0.6abc	5.0 ± 0.7bc	5.4 ± 1.0c	Od
Test 7 (May 19–30)								
12	6.0 ± 1.4b	Oc		16.1 ± 3.2a			6.5 ± 1.7b	Oc
Test 8 (May 31–June 12)								
15	8.8 ± 1.4b	Oc		16.1 ± 2.6a			7.2 ± 1.4b	Oc

[a] Means within tests with different letters are significantly different, $P < 0.05$, Duncan's multiple-range test (Duncan, 1955).
[b] All septa were loaded with 200 μg of their respective blends except as noted. The composition of each blend and release ratios are given in Table 2.

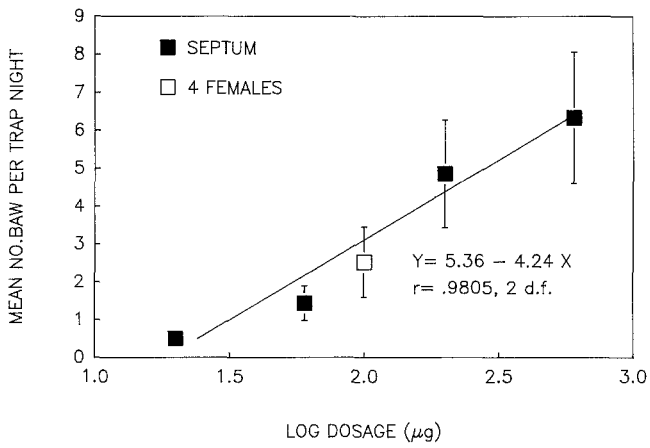


FIG. 1. Capture of BAW males in bucket traps baited with different doses of the three-component pheromone blend on rubber septa (B3-2: 2.5% Z9–14:OH + 87.2% Z9,*E*12–14:AC + 10.3% Z11–16:AC).

The results presented here prove conclusively that Z9–14:OH, Z9,*E*12–14:AC, and Z11–16:AC are active components of the sex pheromone blend emitted by beet armyworm females, and that the first two components are required for capture of beet armyworm males in the field in Florida. It is possible that differences in pheromone blends exist among strains from different geographical areas. However, previous studies have shown that it is possible to capture males of a species with an incorrect or incomplete pheromone blend or even with an analog of a pheromone component (Tumlinson et al., 1986), particularly when massive doses are used. Thus, for accurate analysis of the effectiveness of a synthetic blend, it must be dispensed at approximately the same rate and the same ratio that the natural blend is dispensed by the females. The three-component blend that was most effective in our studies should be effective at doses of 200 μg/septum for detection and monitoring of beet armyworm populations in most areas where this pest occurs. It remains to be seen whether or not a blend of the five components released by the female will be more effective than the three-component blend in traps of a different design. More behavioral experiments are needed to elucidate the role of Z9–14:AC in the communication of this species.

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ISOLATION OF CORN SEMIOCHEMICALS ATTRACTIVE AND REPELLENT TO WESTERN CORN ROOTWORM[1] LARVAE

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Abstract—Dichloromethane extracts of germinating corn are significantly attractive to western corn rootworm larvae in choice tests with equal levels of carbon dioxide present on both sides of the choice. Two fractions that are significantly attractive and two fractions that are significantly repellent to larvae were isolated from these extracts of germinating corn by gas chromatography and silica gel chromatography. In a separate set of experiments, Porapak N was used to collect headspace volatiles from germinating corn; significantly more larvae were attracted to aliquots of these extracts in single-choice tests without added carbon dioxide present than to solvent controls.

Key Words—Coleoptera, Chrysomelidae, *Diabrotica virgifera virgifera*, semiochemical, kairomone, attractants, repellents, *Zea mays*, western corn rootworm.

INTRODUCTION

More acreage is treated with insecticide for *Diabrotica* spp. than for any other insect pest in the United States (Suguiyama and Carlson, 1985). An estimate of crop losses and treatment costs for corn rootworms is in the range of $1 billion per year (Metcalf, 1986). Because of their economic importance, the chemical

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ecology of adult *Diabrotica* has been well studied. The sex pheromones, as well as many attractants and feeding stimulants, have been identified for adult beetles (Guss et al., 1982; Chuman et al., 1987; Lampman and Metcalf, 1988; and references therein). However, it is the larval stage of most *Diabrotica* spp. that is damaging. The only compound known to have behavioral importance to any *Diabrotica* spp. larvae is carbon dioxide, which is an attractant for larvae of the western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte (Strnad et al., 1986; Strnad and Bergman, 1987; Hibbard and Bjostad, 1988). WCR larvae are oligophagous and feed only on certain species of Poaceae (Gramineae) (Branson and Ortman, 1967, 1970). Because carbon dioxide is released by the roots of most plant species (Harris and Van Bavel, 1957), it does not appear to provide a basis for the ability of the larvae to distinguish the roots of host plants from the roots of nonhost plants. Hibbard and Bjostad (1988) collected corn seedling volatiles and demonstrated that these volatiles in conjunction with carbon dioxide were significantly more attractive to WCR larvae than the same level of carbon dioxide alone. The aim of the present study was to isolate these corn semiochemicals.

Two separate fractionation procedures were used to isolate the compounds of interest from the attractive crude extract. The first involved the use of silica gel column chromatography, which separates primarily on the basis of polarity. The second involved gas chromatography, using a column that separates primarily on the basis of molecular weight. A sequential fractionation scheme modified from Silverstein et al. (1967) then was used for isolating the individual semiochemicals. Behavioral choice-test bioassays were used to test the biological activity of crude extracts and individual fractions.

METHODS AND MATERIALS

Larvae and Corn Source. A nondiapausing strain of *D. virgifera virgifera* was obtained in June 1986 from the USDA-ARS laboratory in Brookings, South Dakota. The rootworm colony was maintained with the methods of Jackson (1986) as modified by Hibbard and Bjostad (1988). Second instars (6–7 days old and 5–8 mm long) were chosen for assays because preliminary work indicated that their behavior was similar to that of unfed, newly hatched first instars (the instar responsible for host location), and second instars were more robust for the large number of bioassays required. Dried, whole-kernel field corn (3090, Pioneer Hi-Bred International, Inc., Johnston, Iowa) not treated with fungicide or insecticide was washed and soaked (Hibbard and Bjostad, 1988), and corn seedlings [pre-stage 0 germinated seed (Hanway, 1966)] were removed after three to five days to obtain corn semiochemicals.

Bioassays with Porapak-Collected Corn Volatiles. Thermally conditioned Porapak N (1 1/2 g) was used as a solid adsorbent (Byrne et al., 1975) to collect

volatiles from approximately 500 g of corn seedlings. A vacuum pump (flow rate 6000 ml/min) pulled room air through the corn and Porapak N for 12 h. The Porapak N was extracted with 4 ml of dichloromethane, and aliquots of this solution (100 μl) were placed on rolls of filter paper (4 $\times$ 4 cm before rolling). The bioassay apparatus (diagrammed in Figure 1A) and procedures are described in detail in Hibbard and Bjostad (1988). Briefly, the solvent was

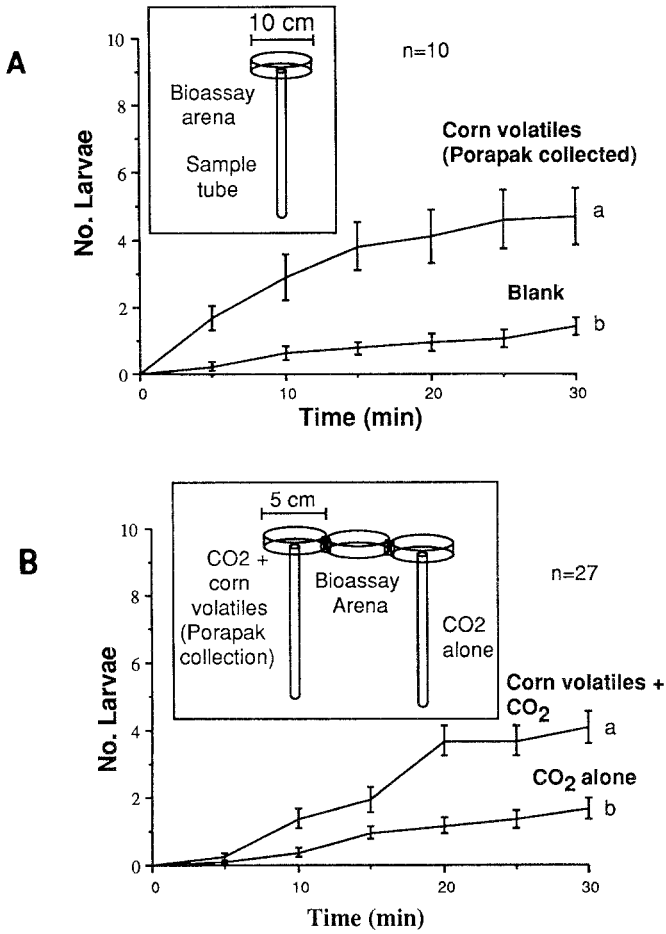


FIG. 1. (A) Single-choice tests with Porapak-collected corn volatiles and blank tubes. (B) Double-choice tests with Porapak-collected corn volatiles with added carbon dioxide on one side versus an equal level of carbon dioxide alone. The number of replicates are indicated by an "n," and diagrams of the bioassay arenas are shown. Standard error bars for each point are indicated. Different small letters represent significant differences ($P < 0.05$) within each graph.

allowed to evaporate, and the rolls of filter paper were placed in the bottom of a clean glass tube (35 cm × 12 mm) that was closed at the bottom. The tube was then connected to the bottom of a plastic Petri dish with a Teflon connector. No carbon dioxide was added. After a 5-min delay to allow for the establishment of a diffusion gradient, 10 larvae were added around the periphery of the Petri dish. Bioassays were conducted under dim lighting. The number of larvae that entered the tube was recorded at 5-min intervals for 30 min. Clean tubes were used as controls.

Compounds of smaller molecular weight (such as carbon dioxide) pass through Porapak (Byrne et al., 1975; Hibbard and Bjostad, 1988), so its use to recover volatile semiochemicals is limited to compounds of higher molecular weight. Because carbon dioxide is a known attractant for WCR larvae (Strnad et al., 1986; Hibbard and Bjostad, 1988), a double-tube choice-test (diagrammed in Figure 1B) was used to assess the attractiveness of carbon dioxide plus the higher-molecular-weight volatile components collected by Porapak to carbon dioxide alone. Equal amounts of carbon dioxide were introduced into both tubes to give a concentration of 5 mmol/mol at bioassay initiation and 1 mmol/mol after 30 min (Hibbard and Bjostad, 1988). The carbon dioxide concentration was measured with an infrared gas analyzer (IRGA, Beckmann model 865) that we interfaced with a Porapak N gas chromatograph column (3 mm × 2 m) as described in Hibbard and Bjostad (1988). A filter paper roll (5 cm diam.) was treated with an aliquot (100 μl) of Porapak-collected volatiles, and a second filter paper roll was treated with distilled dichloromethane as a control. The solvent was allowed to evaporate before placing the rolls in the two sides of the bioassay apparatus. After a 5-min delay, 10 larvae were placed in a small plastic Petri dish lid (40 mm diam. × 5 mm high), and this small lid was placed inside the center Petri dish of the bioassay apparatus. Bioassays were performed in dim lighting, and the number of larvae in each of the dishes was recorded every 5 min for 30 min.

Bioassays with Organic Solvent Extracts of Cryogenically Collected Corn Volatiles. Cryogenic collections of corn seedling volatiles are attractive to WCR larvae and were carried out as previously described (Hibbard and Bjostad, 1988). For these tests, however, 4-ml extracts (pentane or dichloromethane) of these collections were made by rinsing the tubes with solvent. The bioassay procedures were as in the Porapak experiments described above, except that glass wool served as the evaporative surface for the 4-ml collections instead of filter paper. Equal amounts of glass wool (0.10 g) and solvent were used on both sides of the choice test, but one side contained corn volatiles. Carbon dioxide concentrations were 5 mmol/mol at bioassay initiation on both sides of the choice test as described above. The number of larvae that entered either of the end dishes were recorded after 30 min and 60 min. Bioassays were performed in

the dark with a brief interruption after 30 min. New apparatuses were used for each treatment to minimize effects of contamination from the plastic Petri dishes.

Bioassays with Organic Solvent Extracts of Corn Seedlings. Approximately 80 g of moist corn seedlings [pre-stage 0 (Hanway, 1966), 3–5 days old] were extracted by placing them in a glass seed-holding tube (30 cm × 30 mm, tapering to 12 mm) for 3–6 hr and then dripping solvent (pentane or dichloromethane) through them until 4 ml of solution had been collected. Bioassays were as described above, but with new apparatuses to minimize effects of contamination of the plastic Petri dishes.

Silica Gel Column Chromatography. Organic extracts of corn seedlings (4 ml dichloromethane) were evaporated to 0.5 ml in a nitrogen stream. A 22.9-cm pipet (Pasteur type, Curtin Matheson Scientific, Inc.) was cut to 10.5 cm (the location where the pipet tapered) and plugged with glass wool. Silica gel (60–200 mesh, J.T. Baker Chemical Co., Phillipsburg, New Jersey) was transferred to the column (pipet) in dichloromethane until it was 3.5 cm from the top. Crude corn extract (0.5 ml) was then placed at the top of the column keeping the solvent level above the silica gel. Solvent was allowed to pass through the column (and corn extract), and fractions were collected. Because pentane extracts were not biologically active to WCR larvae, the initial solvent system used was 100% dichloromethane. Fractions 2, 3, 4, 5, and 6 were 4 ml each of 3%, 10%, 25%, 50%, and 100% diethyl ether in dichloromethane that had passed through the column. A small portion of each fraction from each replication was analyzed by gas chromatography. Bioassays were as described above, but with new apparatuses to minimize effects of contamination from the plastic Petri dishes.

Gas Chromatography. Organic solvent extracts of corn seedlings (4 ml dichloromethane) were evaporated to less than 10 μl with a nitrogen stream. The highly concentrated solution was separated with an OV-101 packed column (3% OV-101 on Gaschrom Q, mesh 100–120, Alltech Associates Inc., Applied Science Labs, Deerfield, Illinois) of glass tubing (1.83 m × 2 mm ID) in a Hewlett-Packard 5890 gas chromatograph temperature programmed from 60°C to 260°C at 10°C/min with a 1-min initial time and a final time of 9 min. With the detector and noncolumn gases turned off, fractions were collected with clean pipets that were snugly connected to the end of the column with a specially fitted Teflon connector. The collection efficiency of this system ranged from 70% to 85% with known compounds with similar retention times.

Because the attractive fraction from silica gel chromatography contained only one peak, we first isolated this fraction with gas chromatography by collecting during its retention time (16–17 min). The transfer pipets were rinsed with 200 μl of dichloromethane, and bioassays were as described above (with new apparatuses). As with the silica gel fractionation, a full set of fractions also

were collected and bioassayed. Fractions were collected every 3 min during 30 min GC run (all detectable peaks eluted during this time) for a total of 10 fractions. In a second set of GC collections, fractions were collected every minute during the retention time of the attractive fraction 8 (21–24 min) of the 3-min collections. One microliter of the 200-μl rinse of each fraction was analyzed by gas chromatography under the same conditions as the collection for both 3-min and 1-min collections to verify separation of the highly concentrated extract. Further fractionation of the repellent fractions in the 3-min collections were not performed because our primary interest was in host location. Bioassays were as described above, but with new apparatuses to minimize contamination from the plastic Petri dishes. When an isolation on a OV-101 packed column indicated only one peak present, the fraction also was injected onto a methyl silicone capillary column (30 m, RS-150, Supelco Inc., Bellefonte, Pennsylvania) to help determine if the peak had indeed been isolated to one compound.

Statistical Analysis. The statistical program MSTAT (Department of Crop and Soil Sciences, Michigan State University, East Lansing, Michigan 48824) was used for all computerized statistical calculations. Differences between the number of larvae in the two sides of the choice tests were used in ANOVAs for these paired data, while raw data were used for single-choice tests. All data were analyzed with one way analysis of variance (ANOVA). Ninety-five percent confidence intervals were then calculated using the error mean square from the ANOVA table (Snedecor and Cochran, 1980) for data sets with more than two means. Confidence intervals were calculated for fractionation experiments instead of multiple-range tests, because the only comparisons of biological relevance were the percent response of the larvae to zero (see Figures 4 and 5 below).

RESULTS

Significantly more ($P < 0.01$) WCR larvae were attracted to volatiles collected from corn seedlings on Porapak N than to sample tubes with ambient air in single-choice bioassays (Figure 1A). Porapak does not collect small compounds such as carbon dioxide (Byrne et al., 1975; Hibbard and Bjostad, 1988), so the level of carbon dioxide in the Porapak-collected volatile tubes was the same as that in ambient air (verified with infrared gas analysis). These data represent the first indication that carbon dioxide may not be a required component in a blend of corn semiochemicals that is attractive to WCR larvae.

When given a choice between Porapak-collected corn seedling volatiles and blank tubes with an equal concentration of carbon dioxide, significantly more ($P < 0.05$) WCR larvae chose the side with corn volatiles with added

carbon dioxide than the side with carbon dioxide alone (Figure 1B). The difference was statistically significant, but it was quite small compared to this difference when volatiles were collected cryogenically (see Hibbard and Bjostad, 1988).

There was no significant difference between the number of larvae attracted to the side with pentane extracts of cryogenic collections of corn volatiles (with added carbon dioxide) and the number of larvae on the side with an equal level of carbon dioxide alone (Figure 2A). Extracts of germinating corn with pentane also resulted in no significant differences in choice tests (Figure 2B). Pentane apparently does not extract the behaviorally active compounds. However, when dichloromethane was used, significantly more WCR larvae chose the side with extracts of both cryogenic corn collections (Figure 2C, $P < 0.01$) and extracts of germinating corn (Figure 2D, $P < 0.01$).

In choice-test bioassays from silica gel chromatography, fraction 2 (3% diethyl ether in dichloromethane) was significantly repellent ($P < 0.05$), and fraction 4 (25% diethyl ether in dichloromethane) was significantly attractive ($P < 0.05$) (Figure 3). Because GC analysis was performed on each fraction of each replication, an indication of the consistency of separation between individual fractionations was possible. A typical separation is shown in Figure 4. The attractive fraction 4 appeared to contain only one major component. Some variation was observed, and particular replicates in which fraction 5 was attractive contained more of the main component observed in GC analysis of fraction 4 than did other replicates of fraction 5.

Using gas chromatography, we were successful in isolating the attractive peak from silica gel fraction 4 (see Figure 7B below). The fraction was isolated using two different chemical techniques and was a single peak on OV-101 as well as on our methyl silicon capillary column, so we make the assumption that it is a single compound. The compound was significantly attractive ($P < 0.01$) in choice tests with equal levels of carbon dioxide on both sides of the test. Separations by gas chromatography with fractions collected every 3 min resulted in two fractions that were significantly repellent and one fraction that was significantly attractive (Figure 5). Fraction 9 (GC retention time 24–27 min) was significantly repellent, and GC retention times for the major components of this fraction correlated well with the GC retention times of the major components of the repellent fraction 2 of silica gel fractionation. Fraction 4 (GC retention time 9–12 min) was also significantly repellent ($P < 0.05$) and contained only one GC peak on OV-101 and our methyl silicone capillary column (see Figure 7A below), so a repellent was isolated in this 3-min GC collection. Fraction 8 (GC retention time 21–24 min) was significantly attractive in these choice tests, but contained several peaks.

Further fractionation by gas chromatography with fractions collected every

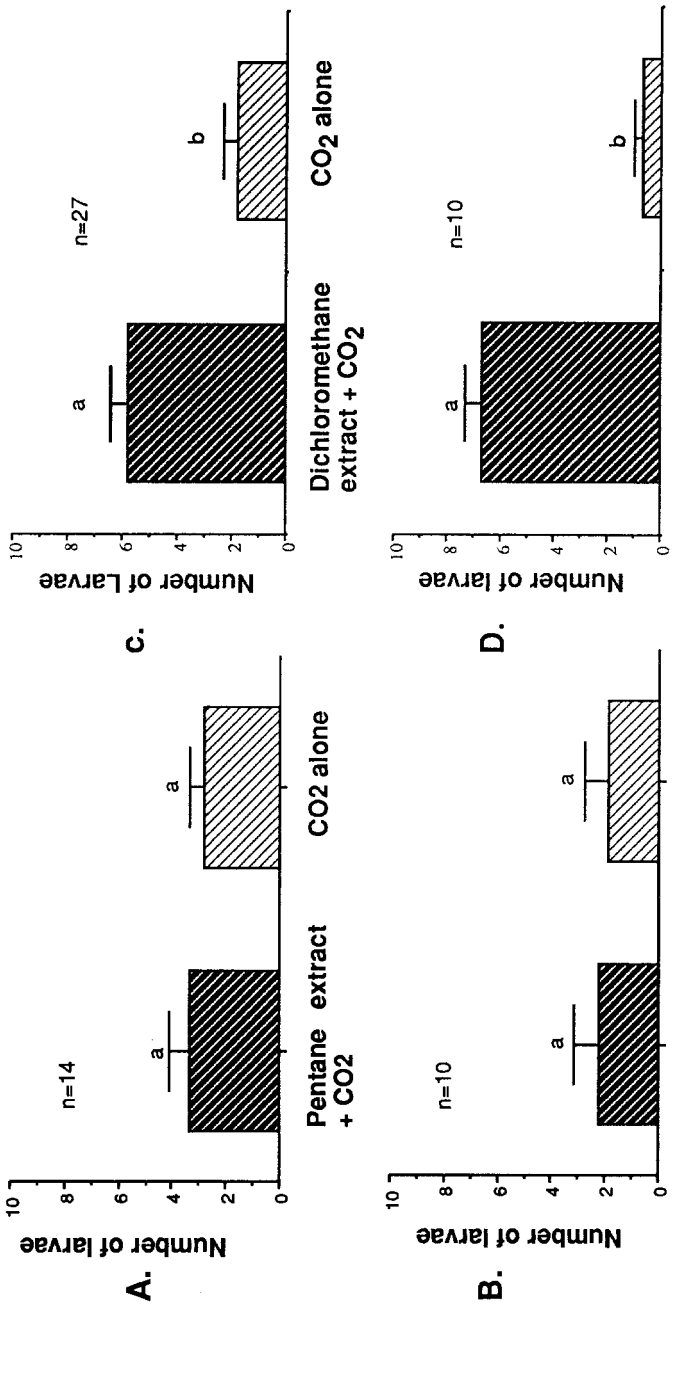


FIG. 2. Choice-test bioassays with carbon dioxide on both sides of pentane extracts of (A) cryogenic corn collections and (B) germinating corn, and dichloromethane extracts of (C) cryogenic corn collections and (D) germinating corn. The number of replicates are indicated by an "n," and error bars represent standard error. Significant differences ($P < 0.01$) within each graph are indicated by different lowercase letters.

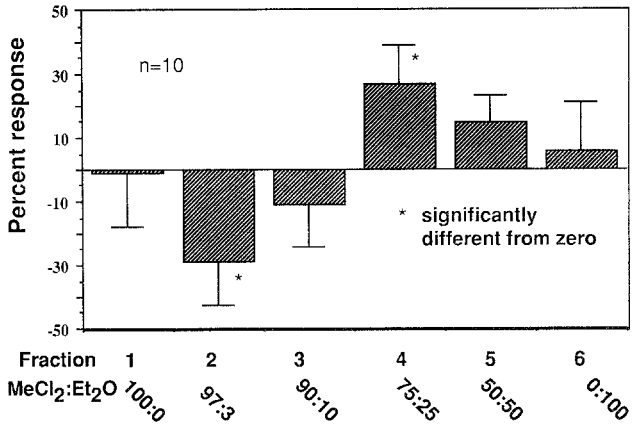


FIG. 3. Choice-test bioassays of silica gel column chromatography fractions of germinating corn extracts. A positive response indicates that more larvae chose the side with corn semiochemicals plus added carbon dioxide than the side with an equal level of carbon dioxide alone. The number of replicates are indicated by an "n," and error bars represent standard error. The only significant differences ($P < 0.05$) indicated are differences from zero, and are designated by an asterisk.

minute during the retention time of fraction 8 above resulted in one fraction (retention time 23–24 min) that was significantly attractive, while the other fractions were not significantly attractive or repellent (Figure 6). GC analysis of this last attractive fraction (retention time 23–24) indicated that at least two major components were present in this fraction (Figure 7C).

DISCUSSION

A number of corn root volatiles have been identified, but have not been tested behaviorally with WCR larvae. Buttery and Ling (1985) used Tenax to collect volatiles from the roots of young (60 cm high) and mature corn plants (2 m high). The principal volatiles recovered were sesquiterpene hydrocarbons: β-caryophyllene, longifolene, bazzanene (tentative), cyclosativene, α-ylangene, and a major hydrocarbon that was not identified. All of these compounds dissolve readily in dichloromethane and should elute with 100% dichloromethane in silica gel column chromatography. In the present study, pentane extracts and the 100% dichloromethane column chromatography fraction had no activity (Figure 3). We used corn seedling [pre-stage 0 germinated corn (Hanway, 1966)] extracts in which corn roots, kernels, and young leaves were present. Compounds previously identified from corn leaves include (Z)-3-hexenol, (Z)-3-

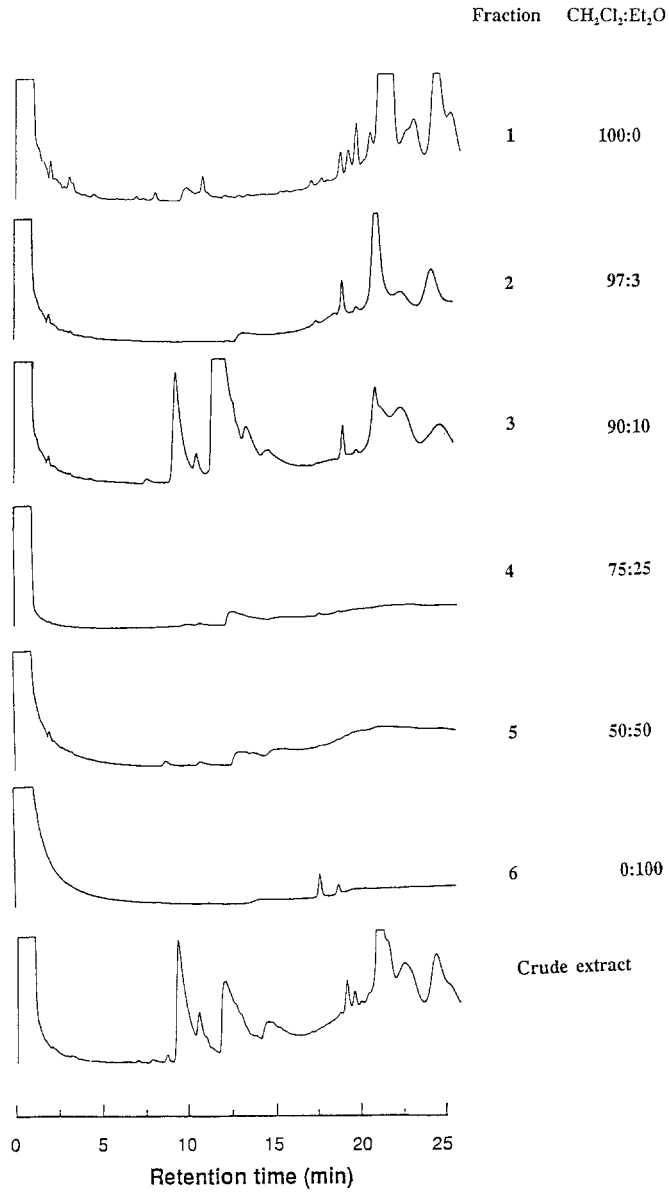


FIG. 4. GC traces on an OV-101 column of a silica gel separation of a dichloromethane extract of germinating corn. The ratio of dichloromethane to diethyl ether that eluted each fraction is given at the right.

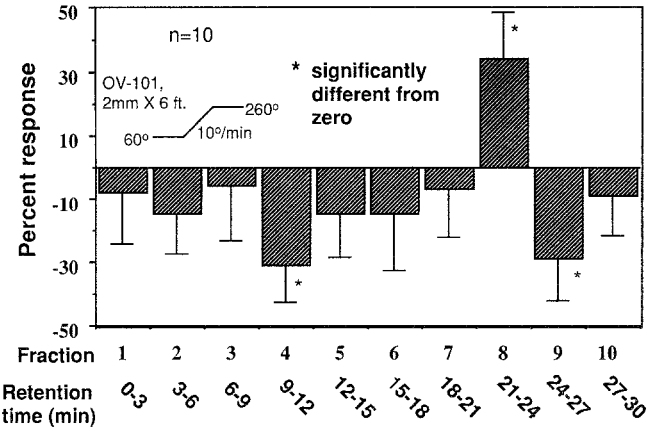


FIG. 5. Choice-test bioassays of gas chromatography fractions of germinating corn extracts. A positive response indicates that more larvae chose the side with corn semiochemicals plus added carbon dioxide than the side with an equal level of carbon dioxide alone. The number of replicates are indicated by an "n," and error bars represent standard error. The only significant differences ($P < 0.05$) indicated are differences from zero and are designated by an asterisk.

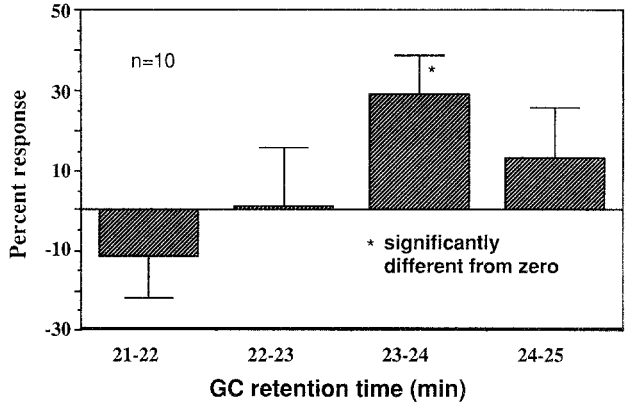


FIG. 6. Choice-test bioassays of gas chromatography fractions of germinating corn extracts. A positive response indicates that more larvae chose the side with corn semiochemicals plus added carbon dioxide than the side with an equal level of carbon dioxide alone. The number of replicates are indicated by an "n," and error bars represent standard error. The only significant differences ($P < 0.05$) indicated are differences from zero and are designated by an asterisk.

hexenyl acetate, (Z)-4-hepten-2-one, (Z)-4-hepten-2-ol, α-ylangene, cyclosativene (tentative), and caryophyllene (Buttery and Ling, 1984). Compounds previously identified from corn kernels include 2-nonan-ol, 2-heptan-ol, 4-hept-en-2-ol, 2-undecan-ol, 4-hept-en-1-ol, 4-hept-en-2-ol, 4-hept-en-2-one, α-ylangene, geranylacetone, β-ionone, and 2,4,7-decatrienal (Buttery et al., 1978). Most of the above corn leaf and kernel compounds would be soluble in pentane and also would be expected to elute with dichloromethane (the first fraction) in silica gel chromatography as well. Therefore, it appears that the attractive compounds from our experiments are not among these previously identified corn volatiles.

In the present study, one compound has been isolated (Figure 7B) that is attractive to WCR larvae in choice tests. As indicated by its elution from silica gel with 25% diethyl ether–75% dichloromethane, this compound is somewhat polar, which is consistent with our earlier observation that dichloromethane extracts are attractive and pentane extracts are not. One additional fraction [which contains at least two GC peaks (Figure 7C)] is also attractive in choice tests (Figure 6). One repellent has been isolated (Figure 7A), and another fraction [which contains at least three GC peaks (Figure 7D)] is repellent as well (Figure 5).

It may seem odd that compounds that are repellent to WCR larvae would be found in corn extracts, especially when the corn extract overall was found to be attractive. However, allelopathic chemicals that act as a defense against herbivores are produced by many plants, including corn. For example, Klun et al. (1967) showed that corn plants produce 2,4-dihydroxy-7-methoxy-(2H)-1,4,-benzoxazin-3(4H)-one (DIMBOA), which is a main factor in resistance of corn to first-generation European corn borer. Other classes of secondary plant compounds produced by *Zea* include alkaloids, cyanogenic glycosides, phenolics, and terpenes (Redak, 1987). All of these classes of compounds have been shown to deter herbivory in other systems. It may be that the repellent compounds we have isolated are secondary compounds produced by corn that are in some way toxic to WCR larvae.

All four of the active fractions in the present study were separated from crude dichloromethane extracts of germinating corn. Attempts were made to keep the concentration in fractionation experiments the same as that in experiments with the attractive crude extracts, but we have evidence that indicates that dosage is an important factor. The attractive compound isolated (Figure 7B) is the major peak in silica gel fraction 4 (Figure 4). However, in GC fractionations, the fraction with this retention time (fraction 6) was not attractive. GC analysis indicated that this peak was present in relatively high amounts in fraction 6 of the GC fractionation (a number of other compounds were also present). As shown in Figure 4, relatively high amounts also occurred in silica

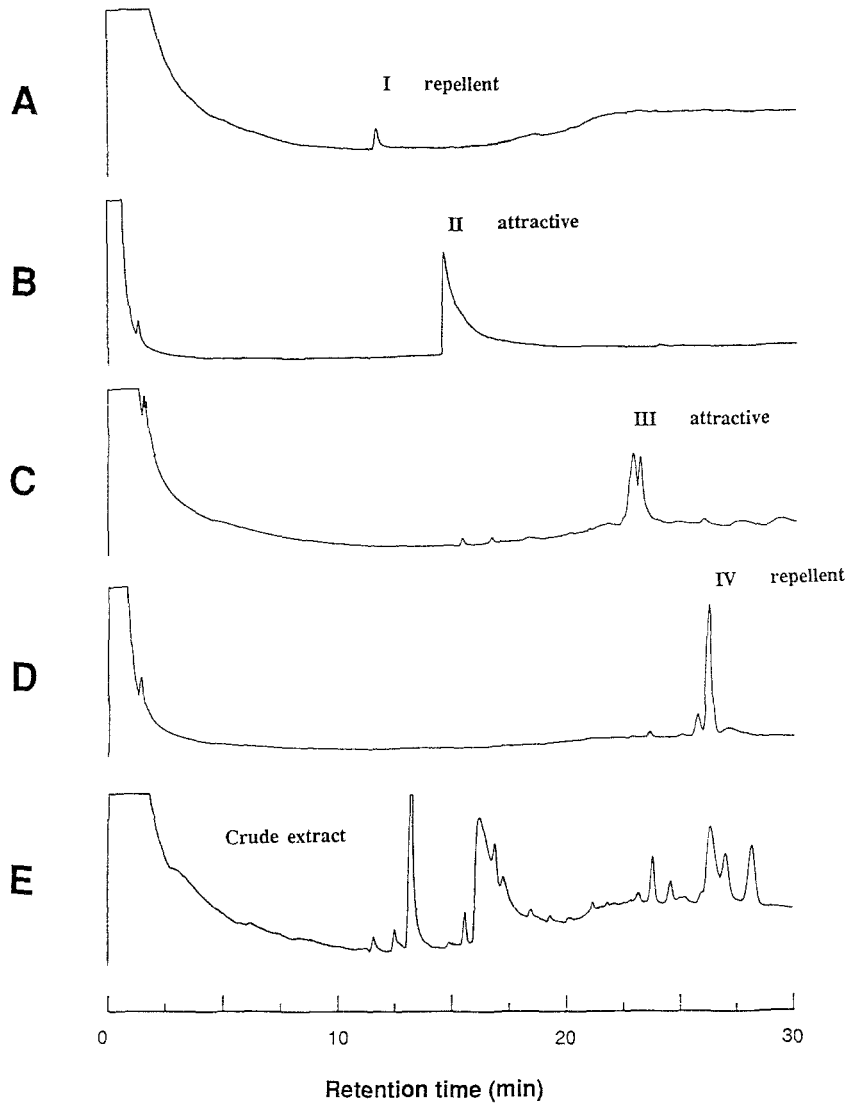


FIG. 7. GC traces of active fractions (doses of active fractions have been concentrated in relation to the crude extract). (A) Three-minute GC collection from a retention time of 9–12 min. (B) One-minute GC collection from a retention time of 16–17 min. The compound isolated was also the major component of silica gel fraction 4. (C) One-minute GC collection of retention time 23–24 min. (D) Three-minute GC collection from a retention time of 24–27 min. The major peaks present in this fraction also were present in silica gel fraction 2. (E) Dichloromethane crude extract of germinating corn.

gel fraction 3 (which was not attractive), while much lower amounts were found in silica gel fraction 4 (which was attractive). We believe that the compound isolated is not attractive in very high doses.

Porapak-collected corn seedling volatiles tested in combination with carbon dioxide were more attractive than a comparable amount of carbon dioxide alone, but the difference between the two sides of the choice test was much less than the difference between the two sides of the choice test in cryogenic corn collections in Hibbard and Bjostad (1988). This indicates that either Porapak N does not collect all of the attractive components from corn that a cryogenic collection does or that the concentration presented to the larvae in the Porapak experiment was not as close to the optimal concentration as was the concentration of these volatiles in the cryogenic collection experiment.

Because of its low molecular weight, carbon dioxide diffuses over greater distances in the soil atmosphere than volatile exudates of higher molecular weight (Green, 1971). Branson (1982) found that WCR larvae are attracted to the roots of nonhost as well as host plants. WCR larvae may be using carbon dioxide as a primary cue for longer distance orientation and more specific cues for close range orientation. Strnad and Dunn (1990) found that when unfed first-instar larvae contacted maize roots, they no longer were attracted to carbon dioxide, but continued localized searching.

Bioassays of Porapak extract in single-choice tests (Figure 1A) indicate that one or more corn semiochemicals are attractive to WCR larvae without carbon dioxide present. These data, in conjunction with the results of Hibbard and Bjostad (1989), suggest that it may be possible to incorporate these larger semiochemicals into insecticide formulations and attract WCR larvae to the insecticide to increase the efficacy of rootworm insecticides.

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ALLELOCHEMICAL REGULATION OF REPRODUCTION AND SEED GERMINATION OF TWO BRAZILIAN *Baccharis* SPECIES BY PHYTOTOXIC TRICHOTHECENES

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Abstract—The potent phytotoxic trichothecene roridins and baccharinoids occur naturally in the Brazilian plants, *Baccharis coridifolia* and *B. megapotamica*. Biosynthesis of roridins in *B. coridifolia* appears to be linked to pollination, and the phytotoxins then accumulate in the seed. The roles of the phytotoxins in pollination, seed maturation, and germination of the *Baccharis* species were investigated. The high production of roridins occurred only in seeds resulting from intraspecific pollination, and the concentration of the toxins in the seeds generally increased with seed maturity. Removal of seed coats from trichothecene-producing Brazilian *Baccharis* species (*B. coridifolia* and *B. megapotamica*) and non-trichothecene-producing American *Baccharis* species (*B. halimifolia* and *B. glutinosa*) resulted in improved seed germination of *B. halimifolia* and *B. glutinosa* but complete inhibition of seed germination of *B. coridifolia* and *B. megapotamica*. Addition of seed coat extracts of the Brazilian *Baccharis* species of dilute solutions (10^{-6} μg/ml) of roridins or baccharinoids to the decoated seeds of *B. coridifolia* and *B. megapotamica* resulted in germination, while seeds of *B. halimifolia* and *B. glutinosa* were killed by the phytotoxins. Roridins interacted with gibberellic acid, a germination promoter, but not with abscisic acid, a germination inhib-

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itor. The results from this study suggest that macrocyclic trichothecenes have a regulatory role(s) on reproduction and germination of Brazilian *Baccharis* species in their natural habitat.

Key Words—allelochemicals, *Baccharis coridifolia*, *Baccharis megapotamica*, Asteraceae, trichothecenes, phytotoxins, pollination, seed germination.

INTRODUCTION

Plants of the genus *Baccharis* are woody perennial shrubs belonging to the family Asteraceae (sunflower family), which is one of the largest angiosperm families. The more than 500 neogeic species of *Baccharis* are confined to the Western Hemisphere except for *B. halimifolia*, which was introduced into several countries by man (Jeffery, 1978). Twenty-one species are native to the United States (Shelter and Skog, 1978), and large concentrations of *Baccharis* species occur in Brazil and in the Andes, indicating this area is the probable center of origin for the genus (Teodoro-Luis, 1955).

Baccharis flowers are usually whitish yellow, dense, and bloom in late spring to summer depending on location and species (Nokes, 1986). Corollas of the pistillate flowers are very slender and threadlike while those of the staminate flowers are larger and usually lobed (Robinson and Fernald, 1908). The pollen grain is usually yellow and spherodial and ranges from 16 to 32 μm in diameter (Jackson, 1975). Because of dioecy, *Baccharis* plants are normally cross-pollinated (Fryxell, 1957). Seeds may be very small, usually ovate in shape with pappus, approximately $2 \times 3 \times 0.25$ mm in size and weigh between 50 and 500 μg, depending on the species (Martinoli, 1940). Total seed production decreases with plant age and germination rates range from 70% to 99% (Panetta, 1979). *Baccharis* plants are usually tolerant to drought and saline soil and have significant beneficial values, e.g. *B. pilularis* is sold as a groundcover or hedge for low maintenance landscape and for soil erosion control (Thompson et al., 1976). Other uses include folk medicine and fragrancies (Boldt, 1989).

Baccharis species produce many secondary metabolites that may have contributed to their evolutionary success by protecting them against herbivores, microorganisms, and other plants (Cronquist, 1981). *Baccharis coridifolia* APDC and *B. megapotamica* Sprengel, have been shown to accumulate substantial amounts of a series of closely related macrocyclic trichothecenes (Habermehl et al., 1985; Kupchan et al., 1977; Jarvis et al., 1987b) that are normally produced by fungi, especially *Myrothecium* species (Ueno, 1983). Trichothecenes are terpenoids and can be divided into two groups: simple trichothecenes (e.g., deoxynivalenol, diacetoxyscripenol, T-2 toxin, etc.) and macrocyclic trichothecenes (e.g., baccharinoids, roridins, verrucarins, etc.).

They exhibit a wide spectrum of biological activity, including antitumor (Kupchan et al., 1977), antimicrobial (Freeman, 1955), and insecticidal (Kishaba et al., 1962) properties. Furthermore, they are known causes for livestock poisoning (Habermehl et al., 1985) and for contact dermatitis in humans (Ueno, 1983). Baccharinoids have been isolated from *B. megapotamica* (Jarvis et al., 1987a; Kupchan et al., 1977), while roridins, miotoxins, and verrucarins have been isolated from *B. coridifolia* (Habermehl et al., 1985; Jarvis et al., 1987b). Trichothecenes, especially the macrocyclic ones, are also highly phytotoxic (Cutler and Jarvis, 1985; Kuti et al., 1987), and their presence in the Brazilian *Baccharis* species is an interesting phenomenon.

Previous investigations on the origin of the macrocyclic trichothecenes in the Brazilian *Baccharis* species revealed no direct involvement of fungi (either symbiotically or endophytically) in the production of these compounds (Jarvis et al., 1987a, 1988a,b), although *M. verrucaria* has been cultured from roots of *B. coridifolia* (Habermehl, 1989). The roridins and verrucarins in *B. coridifolia* were reported to occur only in female plants that have been pollinated (Jarvis et al., 1988a,b), but recent data indicate that these compounds also are present in male plants, although at significantly lower concentrations (Jarvis et al., 1990). Trichothecenes also accumulate (up to 5% by weight) in mature seeds with the highest levels found in seed coats (Jarvis et al., 1988a,b). Of the more than 20 North and South American *Baccharis* species examined so far, macrocyclic trichothecenes have been found only in *B. coridifolia* and *B. megapotamica* (Jarvis et al., 1990). Both Brazilian species (*B. coridifolia* and *B. megapotamica*) are extremely tolerant to trichothecenes, while the North American species (e.g., *B. hamilifolia* L., *B. sarothroides* Gray, *B. glutinosa* Pers., *B. neglecta* Britton, *B. texana* Gray, etc.) are sensitive to macrocyclic trichothecenes (Jarvis et al., 1981, 1988a). Available evidence suggests that the two Brazilian *Baccharis* species are unique in their ability to accumulate these potent phytotoxins and that these compounds may play important role(s) in reproduction of these plants.

Since the level of trichothecenes in *B. coridifolia* is linked to pollination, a study on the involvement of *Baccharis* maternal plant material on trichothecene production and the role of trichothecenes in seed development and germination of the Brazilian *Baccharis* species was done. The objectives of this study were: (1) to determine the inheritance pattern of *Baccharis* plants with respect to trichothecene production by crossing Brazilian species with non-trichothecene-producing North American species, (2) to monitor trichothecene production from pollination to seed maturation, (3) to determine the effects of trichothecenes on germination of intact and decoated *Baccharis* seeds, and (4) to develop a hypothesis to explain the role of trichothecenes in seed development and germination.

METHODS AND MATERIALS

Seed Material. Four *Baccharis* species were used in this study. Two were trichothecene-producers: *B. coridifolia* (BC) and *B. megapotamica* (BM), and two were non-trichothecene-producers: *B. halimifolia* (BH) and *B. glutinosa* (BG). Seeds of BC and BM were collected in Brazil, while seeds of BH and BG were collected in the United States during the summers of 1987 and 1988. Seeds were stored at 4°C, and their viability tested prior to use by the tetrazolium method of Hartman (1981).

Breeding Experiments. Pollen was collected from dehisced anthers of three *Baccharis* species (BC, BH, and BM). Only BC female plants were used in this experiment, since BM plants grown in the greenhouse at the University of Maryland, College Park, have not flowered (Jarvis et al., 1988b). In contrast, BC plants flower every year. Four apparently healthy BC female plants with numerous flowers were selected and 48 flowers were selected randomly and enclosed in nylon mesh bags prior to anthesis. At anthesis, nine flowers on each plant were cross-pollinated by hand with pollen from the three *Baccharis* species. Three female flowers from each species were used for each female plant. Two flowers of each female plant were not pollinated and served as controls. The pollinated and nonpollinated flowers remained bagged during seed maturation. The remaining flowers on each female plant were tagged and left unbagged to allow for bee pollination by adjacent BC male plants. Seeds were first harvested four weeks after pollination and every two weeks up to two months after pollination.

Trichothecene Analyses. Seeds were analyzed for macrocyclic trichothecenes using HPLC procedures described by Jarvis et al. (1988b). Seed viability tests were done as previously described (Hartman, 1981).

Trichothecene Treatments. Seed coats of BC and BM were removed and extracted with 1% acetone. Seed coat extracts plus roridins A + E (5:4, w/w), baccharinoid B4, and T-2 toxin were compared. Each compound was dissolved in 25 μl of acetone and diluted with water to yield a 10^{-6} μg/ml solution, which is not appreciably phytotoxic to plants (Kuti et al., 1987). All solutions were filter sterilized using Nalgene filters prior to use.

Seed Germination. For the germination studies three replicates of 25 intact and 25 decoated seeds of each species were examined. Imbibed seeds were decoated by aseptically removing seed coats with a scalpel and forceps to avoid injury to the embryos. Both intact and decoated seeds then were germinated in 9-cm Petri dishes lined with two Whatman No. 2 filter papers moistened with 4 ml of either solvent (1% aqueous acetone) or solvent plus trichothecenes or seed coat extracts. The percentage of germination in the controls was unaffected by the presence of 1% acetone. The dishes were incubated in a growth chamber at 25 $\pm$ 1°C with 8 hr of fluorescent light daily for seven days. Germination

or when the radicle was visible was recorded daily. In another experiment, solutions of baccharinoid B4 and roridins A + E (in a ratio of 5 : 4) were applied directly to decoated seeds of BC and BM at 12, 24, 36, and 48 hr after seed coat removal. Trichothecenes were applied to decoated seeds at concentrations of 10^{-3}, 10^{-6}, and 10^{-9} μg/ml.

Trichothecene–Plant Hormone Interactions. Experiments were performed to study possible interactions between trichothecenes (baccharinoids and roridins) and the seed germination hormones, abscisic acid (ABA) and gibberellic acid (GA). Solutions (10^{-6} μg/ml) of GA, ABA, and trichothecenes were applied directly to the decoated seeds and the percentage germination determined. Data were significant at the 0.05 level using analysis of varience (ANOVA).

RESULTS AND DISCUSSION

Breeding Studies. There was no significant difference in the ability of cross-pollinated and open-pollinated flowers to set seeds (Table 1). However, only poorly developed ovaries occurred in the unpollinated BC × BC and naturally open-pollinated flowers produced seeds with higher viability than cross-pollinated seeds from the crosses of BC × BM and BC × BH. Germination rate was consistent with viability data. Although, *Baccharis* species appear to be interfertile, compatible pollination may affect seed viability. Trichothecenes were detected in the seeds of self-pollinated BC × BC and open-pollinated BC flowers but not in cross-pollinated BC × BM or BC × BH plants (Table 1, Figure 1). Occurrrence of trichothecenes in seeds of intraspecific crosses suggests that trichothecene production in *Baccharis* species is probably genetically

TABLE 1. SEED VIABILITY, PERCENT GERMINATION, AND TRICHOTHECENE CONTENT OF PROGENY SEEDS OF CROSS-POLLINATED *Baccharis* spp.

Parental cross	Viability (%)	Germination (%)	Trichothecene content (μg/g of seed)[a]
BC × BC[b]	83a[c]	64a	1300
BC × BH	35d	27c	ND
BC × BM	42c	35c	ND
Open-pollinated BC	72b	50b	400
Unpollinated BC	Oe	Od	ND

[a] verrucarins A and J and roridins A and E (see Figure 1); ND: not detectable.
[b] BC = *B. coridifolia*; BH = *B. halimifolia*; BM = *B. megapotamica*.
[c] Mean separation in columns by Duncan's multiple-range test; numbers followed by the same letter are not significantly different at the 5% level.

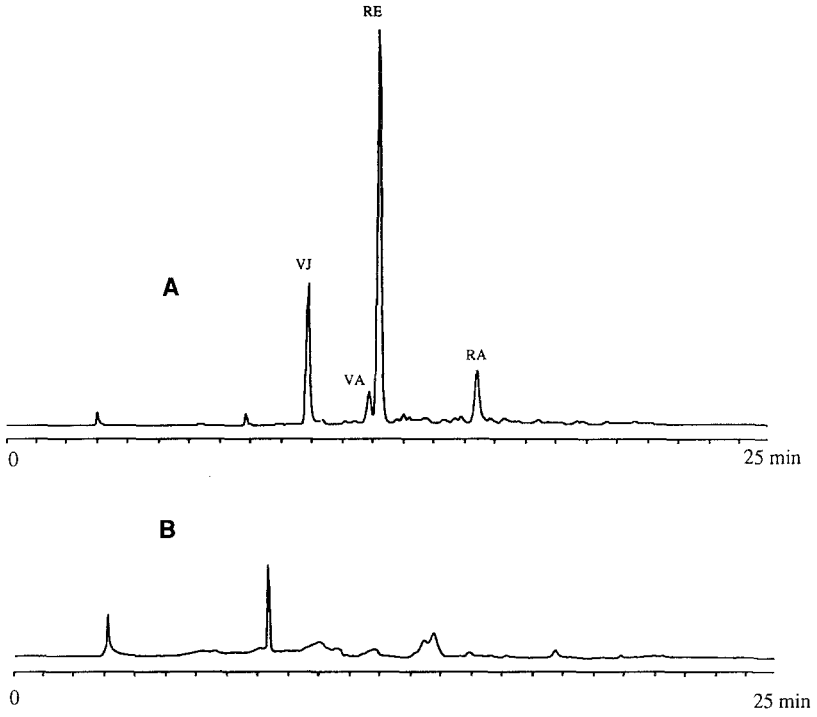


FIG. 1. HPLC trace of *Baccharis coridifolia* seed extracts. (A) Pollinated with compatible pollen (*B. coridifolia*). (B) Unpollinated or cross-pollinated with incompatible *Baccharis* species. VJ, VA, RE, RA = verrucarins J and A and roridins E and A, respectively.

controlled and quantitatively inherited. The cellular locus for trichothecene biosynthesis in *Baccharis* species is not known. Either a search for the enzyme, e.g., trichodiene synthetase (Hohn and VanMiddlesworth, 1986; Hohn and Berenard, 1989) or the use of genetic marker(s) for trichothecene biosynthesis in *Baccharis* flowers would be necessary to elucidate the genetics controlling the trichothecene production mechanism at the molecular level. Levels of trichothecenes in the developing seeds of BC (Figure 2) showed a steady increase in roridins after anthesis, indicating a possible control by roridins of seed development in *B. coridifolia* or, conversely, that seed development regulates the levels of roridins.

Seed Germination. Percentage germination of intact and decoated *Baccharis* spp. seeds is presented in Table 2. Removal of the seed coat improved germinability of BG and BH but inhibited germination of BC and BM. However, addition of BC seed coat extract or roridins A + E reversed this inhibition

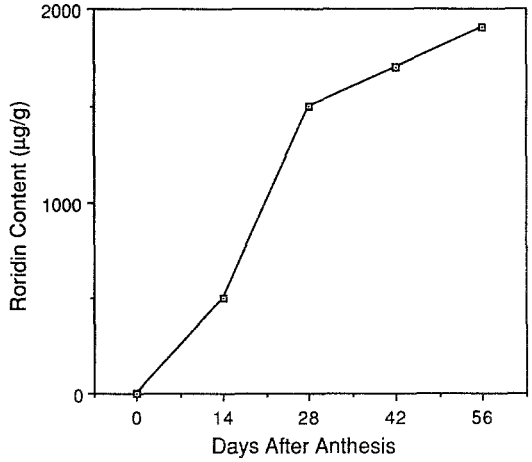


FIG. 2. Total concentrations of roridins A and E in developing *B. coridifolia* seeds.

in BC and to a lesser extent in BM. The addition of BM seed coat extract or of B4, which is the major trichothecene found in *B. megapotamica*, reversed the effect of seed coat removal on the germination of BM but not that of BC. The ability of BM plants to metabolize roridins to baccharinoids (Jarvis et al., 1981) may explain why roridins were able to induce germination in decoated seeds of BM. The addition of T-2 toxin, a simple trichothecene, did not affect germi-

TABLE 2. EFFECT OF SEED COAT REMOVAL AND TRICHOTHECENE TREATMENT ON SEED GERMINATION OF FOUR *Baccharis* spp.

	Germination (%)			
Treatment[a]	BC[b]	BG	BH	BM
Intact + solvent	65a[c]	60b	58b	62a
Decoated + solvent	0b	85a	92a	0c
+ BCsce	62a	0c	0c	42b
+ BMsce	0b	0c	0c	58a
+ RA + RE	63a	0c	0c	34b
+ B4	0b	0c	0c	56a
+ T-2 toxin	0b	0c	0c	0c

[a] Concentration used was 10^{-6} µg/ml; sce = crude seed coat extract; RA + RE is a 5:4 mixture of roridins A and E; B4 is baccharinoid B4.
[b] BC = *B. coridifolia*; BG = *B. glutinosa*; BH = *B. halimifolia*; BM = *B. megapotamica*.
[c] Mean separation in columns by Duncan's multiple-range test; numbers followed by same letter are not significantly different at the 5% level.

nation of either BC or BM. The increase in germination rate of BG and BH seeds when seed coats were removed may be due also to the removal of germination inhibitors present in the seed coat (Anthosen et al., 1970) or it may be due to increasing the availability of oxygen and water to the embryo (Brown, 1940). The response of BC and BM decoated seeds to trichothecene application was time-dependent (Figure 3). BC seed germination was the highest when the roridins were applied immediately after seed coats were removed, while BM seed germination was highest when B4 was applied within 24 hr after seed coat removal. The seeds also responded to the levels of trichothecenes applied (Figure 4): BC seeds germinated best when 10^{-6} μg/ml of roridins was applied compared to 10^{-3} or 10^{-9} μg/ml concentrations. The germination rates of BM

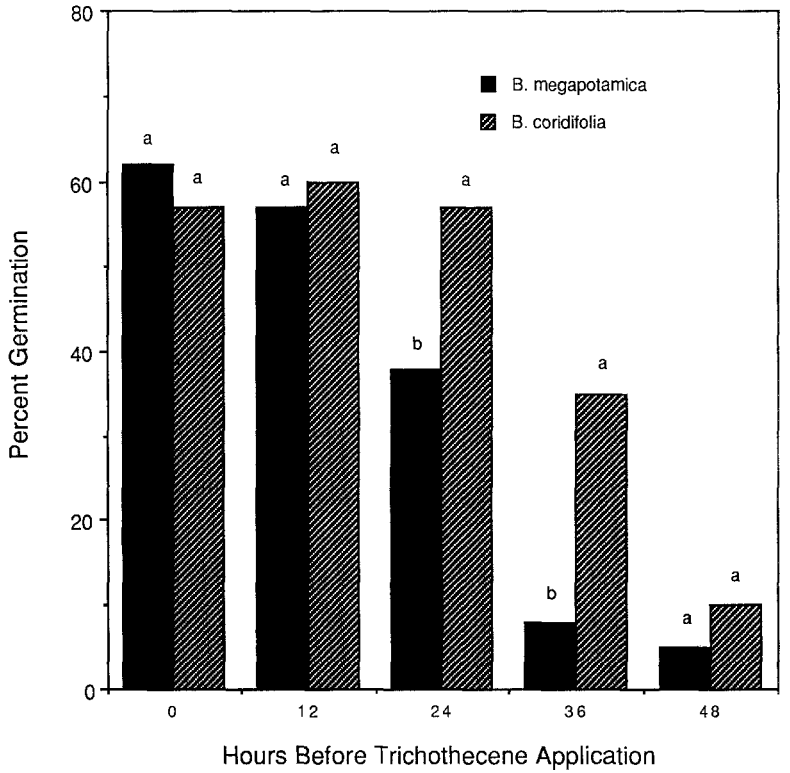


FIG. 3. Effect of time of trichothecene (10^{-6} μg/ml) application on the germination of decoated seeds of *Baccharis coridifolia* and *Baccharis megapotamica*. Means followed by a different letter are significant according to Duncan's multiple-range test at the 5% level.

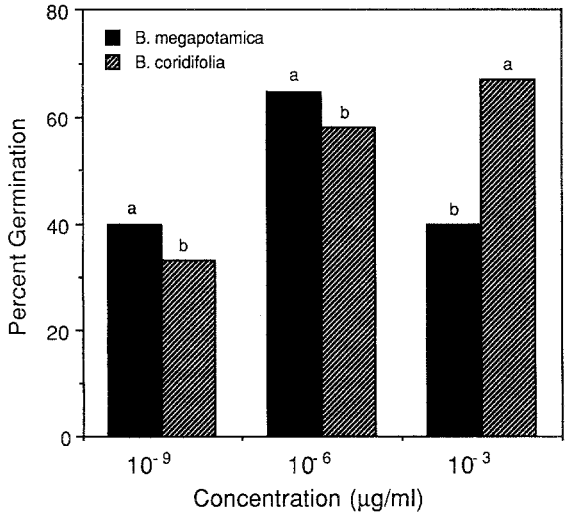


FIG. 4. Effect of trichothecenes on the germination of *B. coridifolia* (roridins A + E applied) and *B. megapotamica* (baccharinoid B4 applied) decoated seeds. Means followed by a different letter are significant according to Duncan's multiple-range test at the 5% level.

seeds increased linearly with increasing concentrations of B4, thus indicating a direct germination–concentration relationship.

Trichothecene–Seed Phytohormone Interaction. The interaction of plant-derived trichothecenes (roridins A + E and B4) with plant hormones (GA and ABA) on seed germination are summarized in Figure 5. BC seeds treated with GA and roridins gave a somewhat higher germination percentage than when decoated BC seeds were treated with the roridins alone (Figure 5G,H), whereas treatment of BM seeds with GA + B4 did not significantly increase seed germination relative to what was observed with B4 alone (Figure 5G,H). ABA appears to have an inhibitory effect on B4 activity on BM seeds, whereas ABA had no effect on roridin-treated BC seeds (Figure 5E). To be able to elucidate the mechanism by which trichothecenes influence *Baccharis* spp. seed germination, one must demonstrate the involvement of trichothecenes in the mechanism(s) that control seed germination. Early studies on the role of seed phytohormones such as gibberellic acids (GAs) and abscisic acid (ABA) in controlling seed dormancy and germination have been reported (Amen, 1968). GAs play a primary role in promoting seed germination only in the absence of inhibitors, while ABA generally inhibits germination and maintains dormancy by reversing GA effects (Khan, 1977). At the molecular level, GAs are known to affect RNA and protein syntheses directly during seed germination (Bewley,

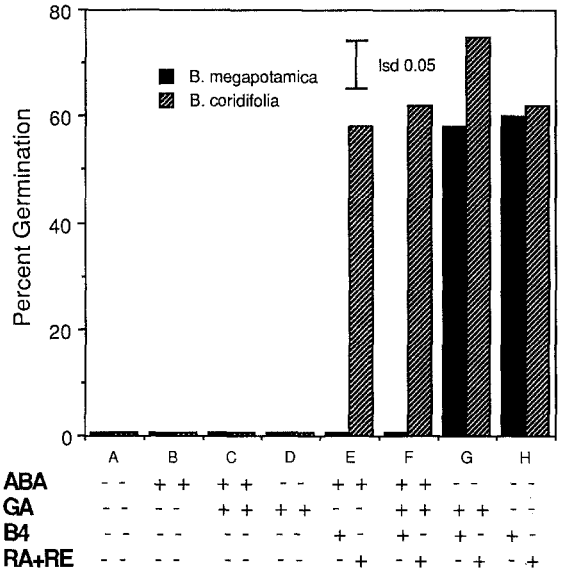


FIG. 5. Effects of trichothecenes (10^{-6} μg/ml) and seed germination hormones (10^{-6} μg/ml) on the germination of *B. coridifolia* and *B. megapotamica* decoated seeds.

1979), and it is known also that trichothecenes commonly bind to susceptible (but not resistant) eukaryotic 60S ribosomes and inhibit peptidyltransferase activity during protein synthesis (Cundliffe et al., 1974). Thus, in BC and BM ribosomes, trichothecenes may not be able to bind to the 60S ribosome (Cannon et al., 1976), which may be why they are not toxic to these plants. The results of this current experiment suggest that GA and ABA may be modulating the ribosomal binding of the trichothecenes. The exact mechanism of interaction between roridins and GA deserves further investigation to help further our knowledge of the subcellular events that occur during germination.

Although, *B. coridifolia* and *B. megapotamica* appear to be unique among higher plants in their ability to accumulate and tolerate phytotoxic trichothecenes, there are significant differences between these species. *Baccharis coridifolia* shows a clear difference between the male and female plants with respect to the concentration of macrocyclic trichothecenes (the levels are 5–10 times higher in female plants), and in the female plants the toxins show a pronounced tendency to accumulate in seeds. On the other hand, *B. megapotamica* shows no measurable difference in the toxin concentration in the male and female plants. Furthermore, seeds of *B. megapotamica* contain relatively low concentrations of trichothecenes (Jarvis et al., 1990). Differences in the two *Baccharis* spp. also can be seen in their responses to various plant hormones that affect

the germination process. For example, it is surprising that ABA has no effect on the germination of roridin-treated BC seeds, but it inhibits germination of B4-treated BM seeds (Figure 5E).

The unique observation reported in this study that BC requires roridins in its seed coats in order to germinate is extraordinary, since seed coats usually contain germination inhibitors, not stimulators (Crocker, 1906; Evenari, 1949). While the level of roridins increases with seed maturation, the level of endogenous GA during seed maturation of *Baccharis* spp. plants should be determined, since the effect of externally applied compounds on plant growth processes is not always convincing evidence for their involvemnet in regulatory mechanisms (Mayer and Poljakoff-Mayber, 1989). It is very difficult to understand what natural selection pressures could have resulted in this novel *Baccharis*–trichothecene system, especially if the source of the trichothecenes is a root-associated *Myrothecium* fungus, as has been suggested (Jarvis et al., 1987b; Habermehl, 1989). An alternative explanation could be that *B. coridifolia* is biosynthesizing these phototoxins directly, which appears to us to be a more likely possibility. The results obtained in this study also show that while interspecific crosses are possible among *Baccharis* spp., trichothecene production occurs only in compatible intraspecific crosses. Additional studies are needed to demonstrate possible maternal influence on trichothecene production in these intraspecific crosses. Increasing accumulation of roridins during seed development and maturation supports the theory that roridins are probably being biosynthesized by *B. coridifolia*. The effects of these trichothecenes on pollination, seed development, and seed germination of the Brazilian *Baccharis* species could have profound implications on the survival of these species in their natural habitat.

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SEX ATTRACTANT PHEROMONE OF DAMSON-HOP APHID *Phorodon humuli* (HOMOPTERA, APHIDIDAE)

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Abstract—Behavioral studies using an olfactometer demonstrated that sexual females (oviparae) of the damson-hop aphid, *Phorodon humuli*, release a pheromone to which males respond. Volatiles produced by the oviparae were analyzed by coupled gas chromatography–single cell recording from the secondary rhinaria on the male antenna and showed the presence of one peak with major activity. Coupled gas chromatography–mass spectrometry suggested a nepetalactol, which was shown to have the 4a*R*, 7*S*, 7a*S* stereochemistry by synthesis from the corresponding nepetalactone isolated from the labiate plant *Nepeta mussinii*. Although the stereochemistry at carbon-1 is not yet established, a synthetic sample comprising ca. 70% 1*S* and 30% 1*R* attracted highly significant numbers of males to water traps placed within and adjacent to a hop garden. Initial studies also indicated attraction of males in both the olfactometer and in the field by volatiles from the primary host.

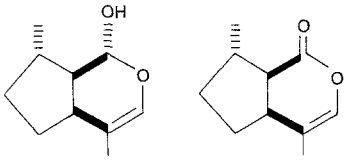
Key Words—Pheromone, sex, attractant, aphid, *Phorodon humuli*, Homoptera, Aphididae, nepetalactol, monoterpenoid, electrophysiology, single cell recording, mass spectrometry.

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INTRODUCTION

Aphids reproduce asexually on their host plants during the summer but many species migrate to winter, or primary, hosts where sexual reproduction occurs. Pettersson (1970, 1971) demonstrated that sexual females (oviparae) of *Schizaphis* spp. attract males by mean of a sex pheromone released from the hind tibiae. Marsh (1972) also demonstrated a sex pheromone for the vetch aphid *Megoura viciae*, and this was recently identified as a synergistic mixture of (−)-(1*R*,4a*S*,7*S*,7a*R*)-nepetalactol (I) and (+)-(4a*S*,7*S*,7a*R*)-nepetalactone (II) (Dawson et al., 1987, 1989).



I II

SCHEME 1.

Electrophysiological recordings from single olfactory cells (SCR) within the secondary rhinaria on the third antennal segments of the male, coupled with gas chromatography (GC), facilitated identification. Two cell types could be distinguished as responding preferentially to compound I or II. The same compounds were found for the sex pheromones of the pea aphid (*Acyrthosiphon pisum*), the black bean aphid (*Aphis fabae*), and the peach-potato aphid (*Myzus persicae*), but in different ratios (Dawson et al., 1990; Hardie et al., 1990). The nepetalactol I alone comprised the pheromone of the greenbug, *Schizaphis graminum* (Dawson et al., 1988).

Aphid sex pheromones have not been shown previously to influence behavior under field conditions. For such investigations, the damson-hop aphid, *Phorodon humuli*, was chosen as a model. The sex pheromone, and chemical components of the host, would be expected to play an important role for this species, since its host alternation is obligate within a narrow range of plants (Eppler, 1986): *Prunus* spp., Rosaceae (winter or primary host) and hops, *Humulus lupulus*, Cannabaceae (summer or secondary host). As the summer host is a valuable crop and control of *P. humuli* by insecticides is now failing because of resistance, use of semiochemicals against this pest should be considered. The sex pheromone of *P. humuli* therefore was characterized chemically and its activity in the field investigated.

METHODS AND MATERIALS

Aphids. Virginoparous *P. humuli* (Badsey strain) from East Malling were maintained on hops (hop progeny 64/84/15) at 20°C with a 16:8 hr day–night regime. Males and gynoparae were produced by transferring virginoparae from the stock culture to 16°C with an 11:13 hr day–night regime. Oviparous females then were obtained by placing gynoparae onto leaves of myrobalan (cherry-plum), *P. cerasifera.*

Pheromone Entrainment. Methods have been described previously (Dawson et al., 1988) and involved entraining volatiles from "calling" oviparae onto Porapak Q and eluting with freshly distilled ether.

Primary Host Volatiles. Twigs or leaves from myrobalan, *P. cerasifera*, were employed in the olfactometer. An ether extract with a concentration of 35 g fresh bark per milliliter was used in laboratory and field studies.

Behavior. Behavioral assays were done in a Perspex olfactometer (100 mm diam.) similar to that described by Pettersson (1970), having a weak airstream directed towards the center from each of four side arms. The odor stimulus (aphids, plant material, or solutions applied to filter paper) was placed at the end of one of the arms. When the stimulus comprised fresh plant material, moistened filter paper was used as a control. Male *P. humuli* (10) were introduced into the center of the arena, and the numbers present in each arm were recorded every two minutes for 20 min.

Coupled GC-SCR. Methods of recording from cells on aphid antennae have been described previously (Wadhams, 1990). For this work, cells associated with the secondary rhinaria on the third antennal segment of the male were employed. GC was done on a 50 m × 0.32 mm id HP-1 fused silica capillary column equipped with a cold on-column injector and with hydrogen as the carrier gas.

Coupled GC-Mass Spectrometry (MS). The methods have been described (Pickett, 1990) and employed the same GC conditions as for GC-SCR. MS was by electron impact at 70 eV, 250°C.

*(4a*R, 7*S*, 7*a*S*)-Nepetalactol (III).* The labiate plant *Nepeta mussinii* was cultivated under glass and, when mature, fresh plants (1,123 g) were macerated in a blender with water (2 liters) and the mixture steam distilled. The distillate was extracted with diethyl ether (2 × 500 ml), the extract dried ($MgSO_4$), filtered, and concentrated to leave a pale yellow oil (1.03 g). This was purified by HPLC using a Jobin Yvon Chromatospac Prep with Lichroprep as the support, eluting with diethyl ether–hexane (12:88), leaving, after concentration, the lactone (570 mg). This was reduced with DIBAH to the (4a*R*,7*S*,7a*S*)-nepetalactol (III) (510 mg, 90%) and shown to comprise ca. 70% 1*S* and 30% 1*R* isomers by nuclear magnetic resonance spectroscopy (Dawson et al., 1989).

The nepetalactol III was stored in glass ampoules sealed under nitrogen and diluted in hexane for laboratory behavioral assays.

Lures. For field trials, pheromone lures were prepared by placing an ether solution of (4a*R*,7*S*,7a*S*)-nepetalactol (10 mg) in closed polyethylene vials (WP/5, Fisons) allowing diffusion through the walls. Myrobalan bark extract (1 ml) was prepared similarly and control lures contained solvent only. Separate vials were used for each test material.

Field Trials. Traps were made from 15-cm-diam. plastic Petri dishes that had been painted on the outside with yellow paint (BS 381C: 1980 Color 309 Canary Yellow), and then with black paint so that the yellow coloring was visible only through the inside. Water containing 0.2% of a nonionic detergent was placed in each dish, and the lures were suspended above the surface of the water by wires. The traps were set on canes 0.6 m above ground level and arranged 2 m apart in six separate randomized blocks. Traps were emptied and the treatments rerandomized daily. Numbers of all morphs of *P. humuli* in the daily trap catches were counted and the data analyzed using a chi-square procedure.

Trapping began on September 12, and initially the blocks were situated outside the hop garden, at least 20 m from the nearest hops. During the week of October 6 to 11, the traps were transferred to within the hop garden (ca. 1 m from the hop bines), where they remained until the end of the trial on November 3. Fresh lures were provided on October 6 and October 17. During the trial, a suction trap (Burkard VI/9, 23 cm fan diam.) sampling approximately 510 m^3 of air per hour (Taylor, 1951) was operated in the hop garden. The inlet was 1.5 m above ground level and the trap was emptied daily.

RESULTS AND DISCUSSION

Results from initial behavioral studies using the olfactometer are given in Table 1. Male *P. humuli* clearly were attracted (or arrested) by a volatile pheromone released by oviparous females, but showed no response to gynoparae. As interspecific attraction has been demonstrated for a number of aphid species (Dawson et al., 1990), the males were also tested against oviparae of *M. viciae*. There was no response, suggesting that the pheromone of *P. humuli* did not comprise either the nepetalactol I or the nepetalactone II.

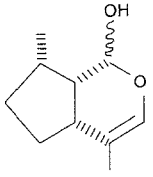
Volatiles entrained from "calling" *P. humuli* oviparae were active on SCR from the male antennae and stimulated only one cell type. The extract was analyzed by coupled GC-SCR and one slightly broadened peak was found to be associated with activity (Figure 1). Coupled GC-MS of the extract showed the compound to have a molecular weight of 168 and a spectrum (Figure 2)

TABLE 1. RESPONSES OF *P. humuli* MALES IN OLFACTOMETER TO FEMALES: CUMULATIVE COUNTS OVER 20 MIN[a]

Stimulus	No. of females	Mean No. males in: Treated arm	Each control arm	Significance[b]
P. humuli oviparae	10	31 ± 1.9	18 ± 1.1	<0.01
	5	33 ± 1.8	17 ± 1.1	<0.01
	1	23 ± 1.2	16 ± 1.0	<0.01
P. humuli gynoparae	3	25 ± 2.7	20 ± 1.1	NS[c]
M. viciae oviparae	10	22 ± 1.7	22 ± 1.0	NS

[a] Number of replicates = 6.
[b] Student's *t* test.
[c] NS = not significantly different at probability level 0.05.

similar to that reported for the nepetalactol I (Dawson et al., 1988). However, the *P. humuli* compound chromatographed later than I, suggesting an isomeric nepetalactol. The stereochemistry at carbon-7 would be fixed early in the biosynthesis and is likely to be the same for all aphids, i.e., 7*S*. Also, since the diastereoisomer of nepetalactol I having the alternative stereochemistry at carbon-1, i.e., (1*S*,4a*S*,7*S*,7a*R*)-nepetalactol, was known to cochromatograph with I (Dawson et al., 1989), then the *P. humuli* compound could only differ from I by having alternative stereochemistry at the ring fusion, i.e., carbons-4a and -7a. The two isomers with *trans*-fused rings would, because of steric strain, open to give iridodials (Dawson et al., 1989), which chromatograph much earlier. This left only one possibility, the all-*cis* (4a*R*,7*S*,7a*S*)-nepetalactol III. This compound previously had been prepared on a very small scale (Dawson et al., 1989) but had not been implicated as a pheromone component for the species studied. Preparation by reduction of the corresponding nepetalactone,



III

SCHEME 2.

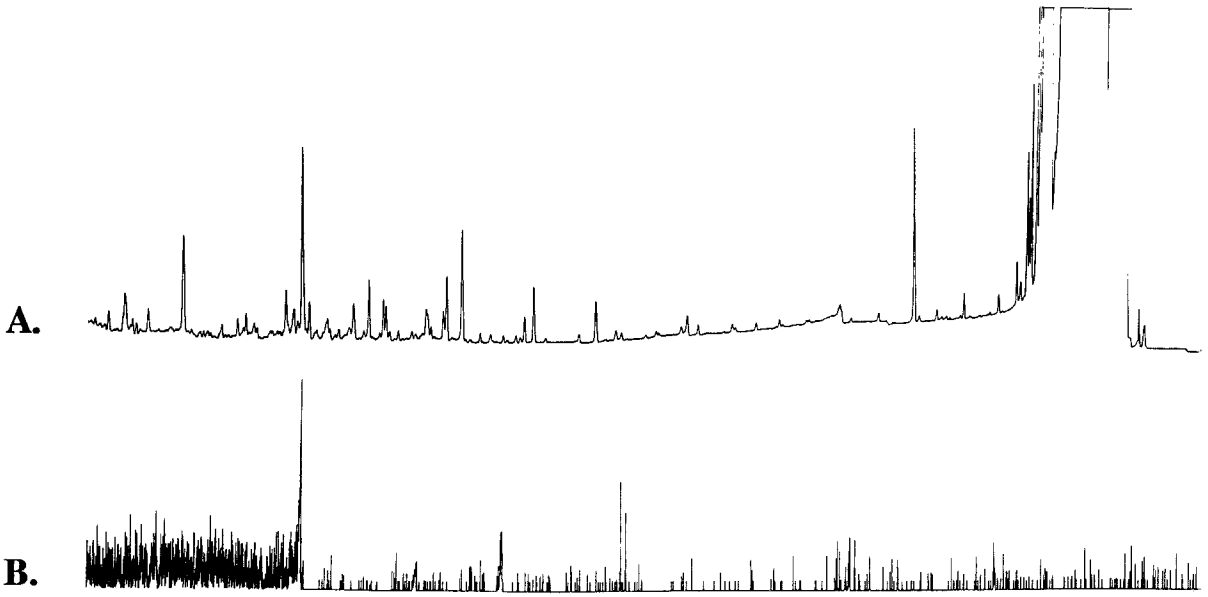


FIG. 1. GC-SCR of volatiles entrained from calling *P. humuli* oviparae. (A) Flame ionization response; (B) action potential summation from cell in secondary rhinarium on third antennal segment of *P. humuli* male.

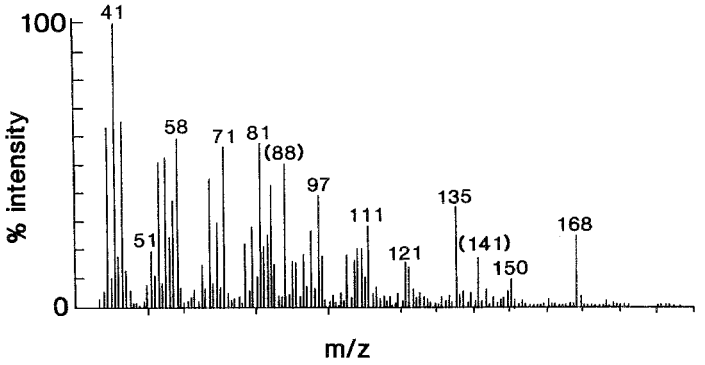


FIG. 2. MS of *P. humuli* sex pheromone; ( ) indicates ions from cochromatographing impurities.

isolated from *N. mussinii*, created a new asymmetric center at carbon-1, with an isomeric composition of 70% 1*S* and 30% 1*R*. The mass spectrum for this product was similar to that obtained by entrainment of *P. humuli*. The isomers were unresolved on GC but, as with the *P. humuli* peak, some peak broadening, indicating partial resolution, was noticed. On coinjection with authentic nepetalactol III, the *P. humuli* peak was enhanced on the HP-1 column and also on a polar column (Carbowax 20 M). This suggested that the pheromone was the nepetalactol III and that the natural material contained both the 1*S* and 1*R* isomers. The nepetalactol III proved to be extremely active in the olfactometer bioassay, attracting males at levels down to 0.15 ng (Table 2).

It has been suggested that sex pheromones of aphids provide only one component of the behavior leading to mate location on the primary host (Steffan, 1987). Since it was considered likely that primary host plant volatiles would

TABLE 2. RESPONSES OF *P. humuli* MALES IN OLFACTOMETER TO NEPETALACTOL III: CUMULATIVE COUNTS OVER 20 MIN[a]

Amount applied (ng)	Mean No. males in: Treated arm	Each control arm	Significance[b]
150	43 ± 2.2	15 ± 1.3	<0.001
15	33 ± 2.5	18 ± 1.0	<0.01
1.5	28 ± 2.3	18 ± 0.9	<0.02
0.15	26 ± 1.0	19 ± 1.1	<0.01

[a] Number of replicates = 6.
[b] Student's *t* test.

TABLE 3. RESPONSES OF *P. humuli* MALES IN OLFACTOMETER TO VOLATILES FROM PRIMARY HOST (MYROBALAN, *P. cerasifera*): CUMULATIVE COUNTS OVER 20 MIN

Stimulus	No. of replicates	Mean No. males in: Treated arm	Each control arm	Significance[a]
Leaf	6	30 ± 2.3	19 ± 0.2	<0.05
Twig	4	35 ± 2.5	18 ± 0.9	<0.001
Bark extract	6	32 ± 2.5	17 ± 1.0	<0.001
Bark extract + nepetalactol III (III as control)	6	36 ± 3.4	14 ± 1.2	<0.01

[a] Student's *t* test.

represent an important olfactory cue in the complex orientation process, some initial investigations were made so that appropriate material would be available for use with pheromone in the intended field trails. Results from olfactometer studies on male response to primary host volatiles are given in Table 3. Positive responses were obtained for myrobalan leaf and twig and an ether extract of bark was shown to be active also. A mixture of bark extract and synthetic pheromone, placed in one arm of the olfactometer, was significantly more attractive than controls comprising the pheromone alone. Other interactions between *P. humuli* and the host plant, and between different morphs, were studied and will be published elsewhere (Pettersson et al., 1990).

In the field trial, when the water traps were placed 20 m outside the hop garden, lures incorporating the nepetalactol III attracted significantly more males than the controls. When the traps were deployed within the crop, the pheromone caught many more males, suggesting that a migratory flight is not essential (Table 4). With fresh lures, the attractancy was enhanced by addition of bark extract. However, synergism was short-lived, which indicated that the compounds involved are either highly volatile or unstable. Daily catches of *P. humuli* in the water traps and in the standard suction trap were also compared (Figure 3). Large numbers of gynoparae were caught in the suction trap in September, but very few were found in the water traps (Table 5). The daily pattern of males caught in both types of trap showed a good correlation. However, each water trap with synthetic pheromone, although having a diameter of only 15 cm, caught as many males as the suction trap which sampled ca. 510 m^3 of air per hour. Indeed, on October 18, water traps incorporating fresh pheromone plus bark extract were three times as effective as the suction trap.

TABLE 4. NUMBERS OF *P. humuli* MALES CAUGHT IN WATER TRAPS IN AUTUMN 1989

	Mean No. males per trap:	
	20 m outside crop	Within the crop (18/10/89)
Nepetalactol III	17.8a	52.0c
Myrobalan bark extract	14.8a	7.8d
III + extract	19.0a	119.5e
Control	2.4b	2.2d

[a] Difference a from b, and c from d from e, $P < 0.05$, based on chi-square test.

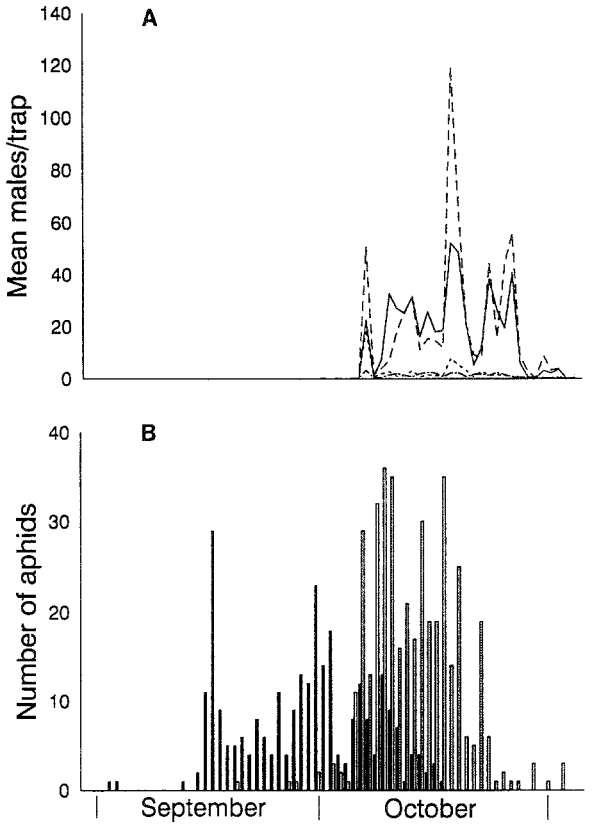


FIG. 3. (A) Daily catches of male *P. humuli* in water traps: ——, nepetalactol III; -----, bark extract; – –, III + bark extract; -·-·-·-, solvent control; (B) daily catches of *P. humuli* in suction trap: ■ gynoparae; ⊡ males.

TABLE 5. TOTAL NUMBERS OF *P. humuli* CAUGHT IN WATER TRAPS IN AUTUMN 1989

	Nepetalactol III	Myrobalan bark extract	III + bark extract	Control
Males	3045	370	3622	210
Gynoparae	74	55	62	53

CONCLUSIONS

The sex pheromone for *P. humuli* is shown to be the (4a*R*,7*S*,7a*S*)-nepetalactol, which we believe has not been detected previously in insects or in plants. The stereochemistry at carbon-1 is not yet defined, but the aphid is thought to produce both 1*S* and 1*R* isomers. A synthetic mixture of these isomers attracted males in the field without the apparent need for other olfactory cues, which is a surprising result in view of previous reports (Steffan, 1987). Nonetheless, there is evidence that primary host volatiles may have a synergistic role that requires further investigation. Now that long-range attraction of male aphids by synthetic sex pheromone has been demonstrated, novel methods of modifying behavior to reduce crop damage can be considered. Although trapping is shown to be feasible, the assertion of Nault and Montgomery (1977) that the pheromone would be more useful in inhibiting mating rather than as a trap lure may yet prove to be correct.

Acknowledgments--We wish to thank L.A. Merritt for preparation of field lures.

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CHARACTERIZATION OF PEDICEL, PAPER, AND LARVAL SILK FROM NEST OF *Polistes annularis* (L.)

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Abstract—The pedicel, nest paper, and larval silk of *Polistes annularis* nests were analyzed by high-resolution solid-state [^{13}C]NMR. The pedicel was found to have a high nitrogen content (11%), and the NMR spectra indicated that it is a mixture of carbohydrate and protein. The pedicel protein has an amino acid composition that is very rich in glycine, alanine, serine, and proline (67% of identified residues), similar to that of some insect silks. Solid-state [^{13}C]NMR indicated that the nest paper is composed predominantly of cellulose. Silk, spun by mature *P. annularis* larvae, was shown by [^{13}C] NMR and amino acid analysis to be a protein very high in serine and alanine (53%), but the amino acid composition is distinct from that of the pedicel protein.

Key Words—CP/MAS [^{13}C]NMR, Hymenoptera, Vespidae, social wasps, *Polistes annularis*, nest pedicel, nest paper, larval silk, scanning electron microscopy,

INTRODUCTION

Social wasps utilize a variety of architectural designs in the construction of their nests (Jeanne, 1975; Edwards, 1980; Akre, 1982; Hansell, 1984; Wenzel, 1989). Many genera of these wasps suspend their nests from a pedicel (petiole)

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built of a tough material primarily composed of an oral secretion (Jeanne, 1975, 1977). Suspension of the nest from the pedicel limits access to the nest by many predators. Several genera of social wasps are known to smear a sternal gland secretion on the pedicel of their nests as a defensive mechanism against predators (Jeanne, 1970, 1979; Hermann and Dirks, 1974; Turillazzi and Ugolini, 1979; Kojima, 1982, 1983; Jeanne et al., 1983; Keeping, 1990). Methyl hexadecanoate has been isolated from the sternal gland of *P. fuscatus* workers, and this methyl ester has been shown to be a repellent to several species of ants (Post et al., 1984; Henderson and Jeanne, 1989). Hexadecanoic acid and octadecenoic acid have been recovered from the pedicel of the nests of *P. annularis* and also may play a role as ant repellents (Espelie and Hermann, 1990). The pedicels of *P. metricus* nests have been shown to have a layer of surface hydrocarbons that are very similar to one another in composition and that may play a role in nest defense (Espelie et al., 1990). The chemical composition of the pedicel, as well as other portions of the nest, have not been examined in detail.

Cross-polarization/magic-angle spinning carbon-13 nuclear magnetic resonance (CP/MAS [^{13}C]NMR) spectroscopy is a relatively new tool that has great potential for the characterization of polymeric materials (Schaefer and Stejskal, 1979; Fyfe, 1983). CP/MAS [^{13}C]NMR is utilized in this report to characterize the pedicel, paper and larval silk of the nest of the social wasp *Polistes annularis*.

METHODS AND MATERIALS

Insect Material. Nests of *Polistes annularis* were collected in Clarke County, Georgia, in June and July 1989. Nests generally had 10–20 workers and 100–200 cells. Pedicels were severed from the nest and their central region (lengthwise) utilized for analysis. Nest paper was taken from cells on the perimeter of the nest, which had not contained mature larvae and did not contain any larval silk. Larval silk was carefully removed from cells in the center of the nest, and care was taken to minimize contamination of the fraction with nest paper. Nest paper, pedicels, and larval silk were extracted with redistilled hexane, air dried, and ground to powders in a centrifugal mill (Brinkmann) with a 40-mesh screen. C, H, and N analyses were performed by Atlantic Microlab, Inc., Atlanta, Georgia. Chitin was purchased from Sigma Chemical Co.

Amino Acid Analyses. Nest paper, nest pedicel, and larval silk samples (10–20 mg) were hydrolyzed in 6 N HCl at 110°C for 24 hr in sealed, evacuated tubes. Amino acids were analyzed with a Microsystems HPLC (Espelie and Kolattukudy, 1985).

Solid-State NMR Spectroscopy. Solid-state NMR spectra were obtained by employing the CP/MAS technique. A Bruker MSL 300 NMR spectrometer

operating at 75.47 MHz for ^{13}C and equipped with high-power amplifiers and a medium-bore (89-mm) probe was utilized for this purpose. The samples were packed in 7-mm Zirconia rotors and were spun at 4.5–5.0 kHz. Data were collected by taking 3K–10K scans using a 4.1-μsec 90° ^{1}H pulse, a 1-msec contact time, and a 1-sec delay time. All spectra were externally referenced to the benzene ring in hexamethylbenzene at 132.3 ppm with respect to tetramethylsilane.

Microscopy. Nest paper, nest pedicel, and larval silk samples were mounted on aluminum stubs and adhered to them with silver paint. A Hummer × Sputter Coater (Anatech) was employed to apply a 50-nm layer of gold–palladium on the samples. A Phillips 505 Scanning Electron Microscope operated at 20 kV was utilized for observing and photographing samples.

RESULTS

The large difference in the amount of nitrogen present in the pedicel and in the paper from the nest of *P. annularis* (Table 1) indicated that the chemical composition of the two portions of the nest are quite distinct, and that the pedicel might contain a biopolymer rich in nitrogen. The recent report (Schremmer et al., 1985) that there is chitin in the salivary secretion of the paper wasp, *Pseudochartergus chartergoides*, suggested that a polymer of *N*-acetylglucosamine might be present in the pedicel of *P. annularis* nests.

CP/MAS [^{13}C]NMR spectroscopy was utilized to examine the composition of the nest pedicel. The CP/MAS [^{13}C]NMR spectrum of the pedicel showed the presence of carbohydrate (Figure 1A). The resonance band at 105.7 ppm is characteristic of the anomeric carbon of a pyranose ring (Heyraud et al., 1979). Additional peaks at 89.5, 84.4, 75.6, 72.8, 65.1, and 62.4 are similar to those previously observed for C_4, $C_{2\text{-}5}$ and C_6, respectively, of cellulose (Himmelsbach et al., 1986). The CP/MAS [^{13}C]NMR spectrum of the nest paper of *P.*

TABLE 1. ELEMENTAL ANALYSIS OF PEDICEL, PAPER, AND LARVAL SILK FROM *Polistes annularis* NEST

	Composition (%)		
	Carbon	Hydrogen	Nitrogen
Pedicel	48.8	7.2	10.9
Nest paper	43.0	6.5	2.8
Larval silk	44.4	7.1	15.3

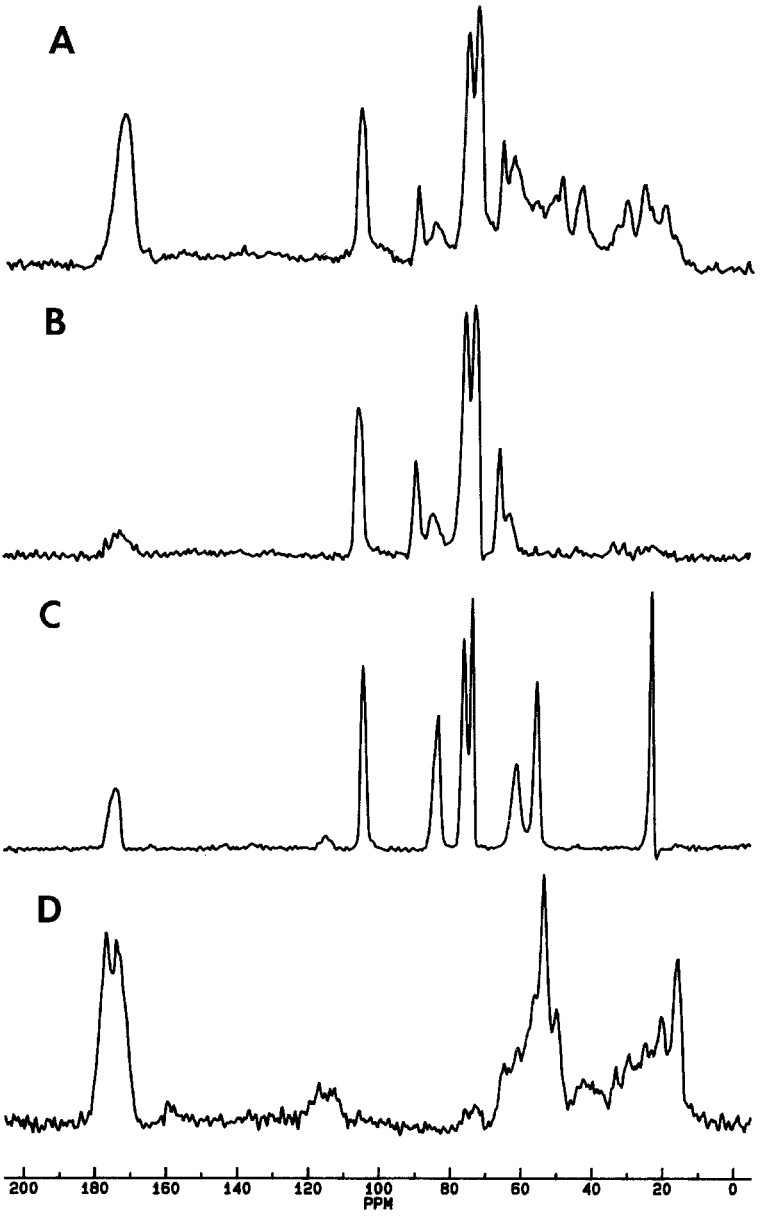


FIG. 1. CP/MAS [^{13}C]NMR spectra of: (A) *Polistes annularis* nest pedicel; (B) *P. annularis* nest paper; (C) a chitin standard; and (D) *P. annularis* larval silk.

annularis nests was dominated by these same resonance bands and appears to be composed primarily of cellulose (Figure 1B). A CP/MAS [^{13}C]NMR spectrum of chitin has peaks from 55 to 105 ppm due to the glucose carbons but is specifically characterized by signals at 23.3 and 173.8 ppm due to the methyl and carbonyl carbons, respectively, of the *N*-acetyl moieties of the polymer (Figure 1C). Since there is a barely detectable peak at 23.3 ppm in the spectrum of the *P. annularis* nest pedicel (Figure 1A), there is very little *N*-acetylglucosamine in this part of the nest.

There is, however, a large carbonyl peak at 172.1 ppm in the spectrum of the pedicel (Figure 1A). The high nitrogen content of the pedicel suggests that this carbonyl peak is due to the presence of protein. A CP/MAS [^{13}C]NMR spectrum of larval silk taken from *P. annularis* nests has major carbonyl peaks at 173.7 and 176.8 ppm and a series of resonances from 15 to 70 ppm (Figure 1D). The pedicel appears to be a mixture of the type of components found in the nest paper and in the larval silk; that is, a combination of cellulose and protein.

The nest pedicel was completely solubilized by treatment with 6 N HCL. Amino acid analysis of the hydrolysate yielded an unusual composition that was dominated by glycine, alanine, proline, and serine (Table 2). The larval silk

TABLE 2. AMINO ACID COMPOSITION[a] OF PROTEIN FROM NEST PEDICEL, NEST PAPER, AND LARVAL SILK OF *Polistes annularis*

Amino acid	Pedicel	Nest paper	Larval silk
Asx	5.4	3.4	6.0
Glx	7.0	4.2	6.5
Ser	10.4	11.1	19.6
Gly	26.4	26.7	7.5
His	0.4	0.6	0.1
Arg	1.4	1.6	8.1
Thr	2.0	2.8	3.1
Ala	17.2	16.2	33.6
Pro	12.5	14.1	0.7
Tyr	0.4	1.1	0.3
Val	6.8	7.3	4.6
Met	0.6	0.4	0.3
Iso	3.1	3.7	1.6
Leu	4.0	4.6	4.5
Phe	0.2	0.8	0.3
Lys	2.0	1.3	3.1

[a] Number of residues per 100 identified amino acids.

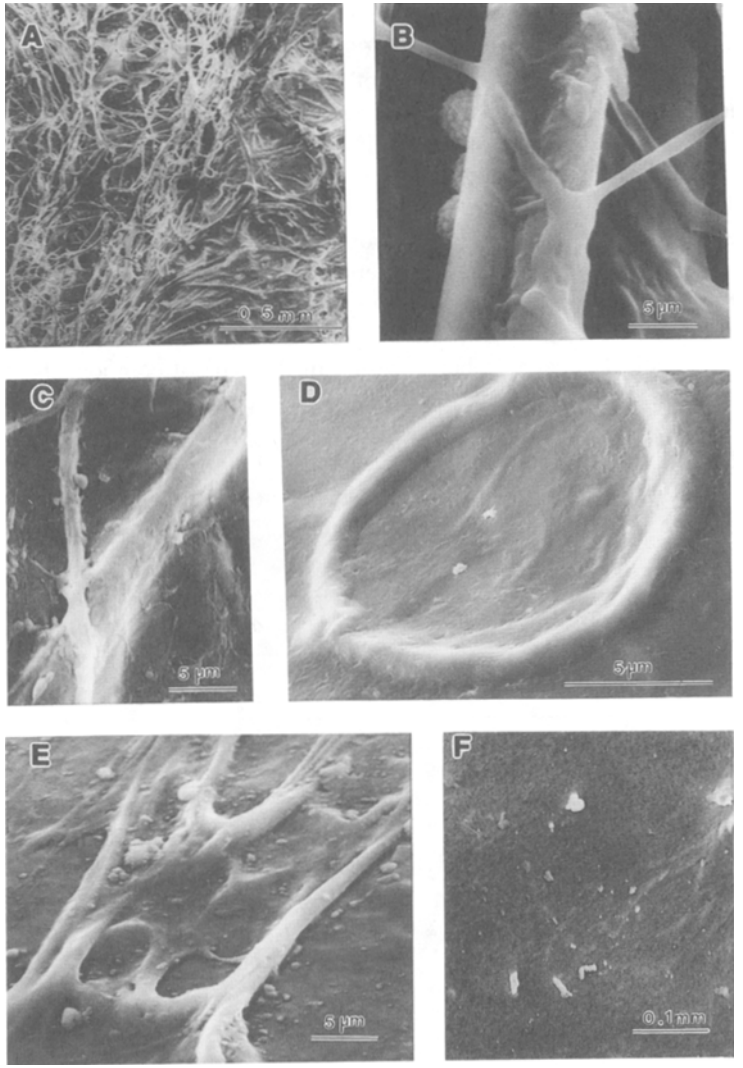


FIG. 2. Scanning electron micrographs of (A and B) the outer surface of the larval silk of *Polistes annularis*; (C) the inner surface of *P. annularis* larval silk; (D and E) fibers on the surface of the pedicel of a *P. annularis* nest; and (F) the amorphous surface of the same pedicel.

had a different amino acid composition with much lower proportions of glycine and, especially, proline. Alanine and serine comprised more than half the residues found in the larval silk protein. Neither hydroxyproline nor hydroxylysine were detected in either the pedicel or larval silk hydrolysates.

The larval silk was examined by scanning electron microscopy (SEM). The outer surface of the silk was covered with a dense array of fibers (Figure 2A). These fibers varied greatly in their diameter (Figure 2B). The inner surface of the silk also had fibers that differed in thickness, but the fibers were not as prevalent, and the larger fibers were not as big in diameter as those seen on the outer surface (Figure 2C). The fibers on the inner surface of the larval silk appeared to be laid down upon and into a relatively smooth amorphous surface. The pedicel of *P. annularis* nests also was examined by SEM. On the surface of the pedicel there were several regions where fibers were visible (Figure 2D and 2E). These fibers were less distinct than those seen on the surface of the larval silk, and they appeared to be embedded into the main amorphous layer. Although these fibers were frequently observed, most of the surface of the nest pedicel was relatively smooth (Figure 2F).

DISCUSSION

The elemental analysis (Table 1), the amino acid analysis (Table 2) and the CP/MAS [^{13}C]NMR spectrum (Figure 1A) clearly show that the pedicel of *P. annularis* nests contains protein. The NMR spectrum of the *P. annularis* larval silk (Figure 1D) is similar to the solid-state NMR spectra previously published for the larval silk of the moths *Bombyx mori* (Kricheldorf et al., 1983; Lindberg et al., 1986; Kramer et al., 1989) and *Antheraea mylitta* (Kramer et al., 1989). The CP/MAS spectrum of the *P. annularis* pedicel (Figure 1A) indicates that it contains cellulose as a major component and a protein that is rich in glycine (43 ppm), alanine (20 ppm), and proline (26, 31, and 49 ppm). *N*-Acetylglucosamine is a minor component that is most likely bound to protein. Chitin is clearly not a major component of the pedicel of *P. annularis* nests. The CP/MAS spectrum of the nest paper indicates that this is primarily cellulose, and this material and the cellulose found in the pedicel are probably derived from plant material, while the protein found in the pedicel and the nest paper are probably derived from the oral secretion of the wasps.

Social wasp larvae produce an oral exudate that is rich in amino acids (Hunt and Noonan, 1979; Hunt, 1982). This exudate, which is presumably produced by the labial gland of the larvae, is imbibed by adult wasps and may be an important energy source for the workers (Maschwitz, 1966; Wilson, 1971). There was very little correlation between the amino acid compositions found for the pedicel or the larval silk proteins (Table 2) and that previously

reported for the larval oral exudate of *P. annularis* (Hunt et al., 1982). The larval exudate did have a high proportion of proline (17% of detected residues), but *P. annularis* larvae had the lowest proportion of proline (range: 17–56%) found for the seven species of social wasps examined (Hunt et al., 1982). When the larvae are mature, the labial gland begins to produce silk and each larva spins a cocoon that completely encloses it within the cell of the nest (Rau, 1929; Ochiai, 1960; Spradbery, 1973; Chao and Hermann, 1983; Ishay and Ganor, 1990).

The pedicel protein has a large proportion of small amino acid residues (Table 2), and the amino acid composition is similar to that found in some insect silks (Lucas and Rudall, 1968a,b; Varman, 1978; Crewe and Thompson, 1979). The relatively large amount of proline (12.5%) in the pedicel protein is higher than that found in most insect silks, but a collagen-like silk with a similar amount of proline has been reported for the cocoon protein of the sawfly, *Nematus ribesii* (Lucas and Rudall, 1968a). Proline is frequently a dominant component of structural proteins (Bursell, 1981; Chen, 1985), and the high proline content of the pedicel protein may help to provide the structural strength required in this part of the nest. Very few adult insects have been reported to produce silk (Rudall and Kenchington, 1971). Among the Hymenoptera, only the adults of *Microstigmus comes* and *Arpactophilus mimi*, social sphecid wasps, have been reported to produce silk, which they use in nest construction (Matthews and Starr, 1984; Matthews and Naumann, 1988).

Although the nest paper of *P. annularis* appears to be predominantly cellulose, the NMR spectrum does have a peak at 172 ppm for carbonyl carbon and a series of small peaks between 20 and 40 ppm that may be due to the presence of protein (Figure 1B). The presence of nitrogen in the nest paper (Table 1) and the amino acid composition of the hydrolysate of the nest paper being very similar to that of the pedicel protein (Table 2), indicates that the wasps apply a proteinaceous secretion to the nest paper that is essentially the same as that used to construct the pedicel. The nest paper of *Polistes fuscatus* was shown by amino acid analysis to have a protein that has serine, alanine, glutamic acid, and proline as the four most abundant amino acids (in decreasing order) (McGovern et al., 1988). This composition is very similar to that found for the nest paper of *P. annularis*, where the most abundant residues were glycine, alanine, serine, and proline (Table 2).

The greater density of fibers seen on the outer surface of the larval silk (Figure 2A and 2B) compared to that found on the inner surface (Figure 2C) may be due to deposition of proteinaceous material on the outside of the cocoon by adult wasps. This possibility might also explain the fact that many of the fibers on the outer surface of the cocoon were larger than those observed on the inner surface. Previous SEM examination, however, has shown great diversity in fiber thickness of the silk produced by an individual insect, and it was sug-

gested by Wallace and Malas (1976) that this was due to the silk being extruded through spinneret grooves of varying diameter. The appearance of the pedicel of *P. annularis*, that is, randomly oriented fibers in an amorphous matrix (Figure 2E and 2F), is very similar to that seen for the material that comprises the surface of the nests of *Pseudochartergus chartergoides* (Schremmer et al., 1985) and *Leipomeles dorsata* (Schremmer, 1983). Future work will examine whether the utilization of similar proteins in construction of the nest is a common feature of social wasps.

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MULTISPECIES TRAPPING OF *Helicoverpa (Heliothis) zea*, *Spodoptera frugiperda*, *Pseudaletia unipuncta*, and *Agrotis ipsilon* (LEPIDOPTERA: NOCTUIDAE)[1]

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Abstract—Multispecies sex pheromone trapping (trapping of more than one species in the same trap) for the bollworm, *Helicoverpa (Heliothis) zea* (Boddie); fall armyworm, *Spodoptera frugiperda* (J.E. Smith); armyworm, *Pseudaletia unipuncta* (Haworth); and the black cutworm, *Agrotis ipsilon* (Hufnagel) was evaluated. Baiting of individual traps with all possible combinations of the four species taken two at a time caused a reduction in the catches of at least one of any two species involved when compared to traps baited for only one species. These results demonstrated the species specificity of sex pheromones identified for these species. Although the effects were reduced in some cases, the interaction between species also was observed when adjacent traps were baited with the sex pheromone dispensers of the different species. These results indicate that the spacing between pheromone traps when more than one species are being trapped at the same site affects trap capture and should be evaluated further.

Key Words—Pheromone, *Helicoverpa (Heliothis) zea*, *Spodoptera frugiperda*, *Pseudaletia unipuncta*, *Agrotis ipsilon*, Lepidoptera, Noctuidae, sex pheromone, trapping, monitoring.

INTRODUCTION

A complex of important noctuid pests of field crops are considered migrants. These include the bollworm (BW), *Helicoverpa (Heliothis) zea* (Boddie) (Hart-

[1] This paper reports the results of research only. Mention of a commercial or proprietary product does not constitute an endorsement by the USDA.
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stack et al., 1982, 1986; Raulston et al., 1990); armyworm (TAW), *Pseudaletia unipuncta* (Haworth) (Steck et al., 1982); black cutworm (BCW), *Agrotis ipsilon* (Hufnagel) (Showers et al., 1989); and fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith) (Pair and Sparks, 1986). We and others are actively studying the migratory behavior and impact of these pests. Sex pheromone trapping is used extensively in these studies. Because using the same trap to monitor activity of more than one species would significantly reduce monitoring costs, tests were conducted to compare catches in traps baited for one species to those in traps baited simultaneously with pheromone dispensers of two species. The effect of baiting adjacent traps with pheromone dispensers of two species also was evaluated. Commercially prepared formulations of the pheromone of the different species are used widely for monitoring and were used in the evaluations.

Coudriet and Henneberry (1976) found that virelure [two-component pheromone blend for the tobacco budworm (TBW), *Heliothis virescens* (F.)], used in combination in the same trap with either looplure or gossyplure did not affect captures of male cabbage loopers (CL), *Trichoplusia ni* (Hübner), or pink bollworm (PBW), *Pectinophora gossypiella* (Saunders), respectively; however, captures of TBW were too low to determine if looplure and gossyplure affected TBW captures. They also reported that looplure in combination with gossyplure did not affect captures of CL, but captures of PBW were significantly reduced. Other studies by Hendricks et al. (1977) reported the feasibility of multispecies trapping involving the CL, soybean looper, *Pseudaplusia includens* (Walker), and TBW.

Limited information is available on multispecies sex pheromone trapping with slow-release dispensers involving the species we studied. Carpenter et al. (1984) reported on the interaction between BW and FAW when dispensers of both were used to bait the same trap; however, the evaluation did not include a more effective pheromone blend formulated in rubber septa developed for FAW by Mitchell et al. (1985).

METHODS AND MATERIALS

All tests were conducted in the Brazos River Valley, Burleson County, in an area of intensive commercial agriculture near College Station, Texas. The major agricultural commodities produced in the area include corn, sorghum, cotton, small grains, and beef.

The sex pheromone dispensers used in these studies are described in Table 1. With the exception of the plastic polyvial dispenser for FAW, all the dispensers were obtained from the indicated commercial sources. The pheromone content of the dispensers was not determined independently, and the information provided was obtained from the commercial source.

TABLE 1. SEX PHEROMONE DISPENSERS USED IN MULTISPECIES TRAPPING STUDIES

Species	Dispenser type	Pheromone blend[a]	Dosage (mg)[a]	Source	References
Helicoverpa zea (BW)	Laminated plastic (1.27 × 2.54 cm)	(Z)-7-hexadecenal (2%) (Z)-9-hexadecenal (3%) (Z)-11-hexadecenal (87%) Hexadecanal (8%)	1.25	Hercon Environmental Co., Emigsville, Pennsylvania 17318	Klun et al., 1980 Lopez et al., 1981 Zvirgzdins and Henneberry, 1983 Lopez and Shaver, 1990
Spodoptera frugiperda (FAW)	Plastic polyvial	(Z)-9-dodecenyl acetate	25.00	not commercially available	Sekul and Sparks, 1976 Mitchell, 1979
	Rubber septum	(Z)-7-dodecenyl acetate (0.45%) (Z)-9-dodecenyl acetate (0.25%) (Z)-9-tetradecenyl acetate (81.61%) (Z)-11-hexadecenyl acetate (17.69%)	2.0	Terochem, Raylo Chemicals, Edmonton, Alberta, Canada T6C 4A9	Tumlinson et al., 1986 Mitchell et al., 1985
Agrostis ipsion (BCW)	Laminated plastic (2.54 × 5.08 cm)	(Z)-7-dodecenyl acetate (75%) (Z)-9-tetradecenyl acetate (25%)	0.80	Hercon Environmental Co., Emigsville Pennsylvania 17318	Hill et al., 1979 Clement et al., 1981
	Rubber septum	(Z)-7-dodecenyl acetate (75%) (Z)-9-tetradecenyl (25%)	0.04	Trecé, Inc. Salinas, California 93915	
Pseudaletia unipuncta (TAW)	Rubber septum	(Z)-11-hexadecenyl acetate (99.2%) (Z)-11-hexadecenal (0.2%) (Z)-11-hexadecenol (0.5%) (Z)-9-tetradecenyl acetate (0.1%)	1.008	Trecé, Inc. Salinas, California 93915	Hill and Roelofs, 1980 McDonough et al., 1980 Farine et al., 1981 Steck et al., 1982 Turgeon et al., 1983

[a] This information was obtained from the commercial source of the dispensers and was not verified independently.

The pheromone trap used in the evaluations was the 75–50 wire cone trap (Hartstack et al., 1979). The traps were installed along fence lines or turn rows either adjacent to, or just within, the borders of corn, cotton, and small-grain fields or pastures. At each trap location, a 1.8-m piece of 1.9-cm-diam. electrical conduit was driven into the ground so that the top of the conduit was ca. 1 m above ground level. The pipe base of the wire cone traps was inserted into the conduit. In corn, the height of the traps was maintained at tassel level. To adjust height in the traps operated in corn, reinforcement bars (1.3 cm diam.) of the proper height were inserted inside the conduit and the top of the reinforcement bar was inserted inside the pipe base of the wire cone traps.

Unless otherwise specified, all evaluations were made using rows of traps with individual or paired traps spaced 50 m apart. In all tests involving three or more treatments, a Latin square in time design was used. Thus, the number of replicates in each test was equal to the number of treatments for each species. When a Latin square design was not used, treatment locations in each replicate were randomized. Treatments were rotated among trap locations within each replicate daily by moving the traps containing the dispensers to prevent contamination of the traps by contact with the dispensers. For each period of days equal to the number of treatments, each treatment was exposed for one night at each location in each replicate.

The interaction of the sex pheromone of the different species was evaluated by baiting the same trap with the sex pheromone dispensers of two different species. Also, the interaction was evaluated when the pheromone dispensers of the different species were placed in separate traps, but the two traps were placed in close proximity (within 1 m of each other). The studies were done with all combinations of four species taken two at a time. Comparisons of male captures of these two treatments were made to captures in traps baited with the pheromone dispensers of the corresponding individual species. To evaluate the treatments, trap lines were established with two trap-mounting bases at each site so that two traps could be operated at any of the sites. Only one site in each replicate had two traps (denoted here as adjacent traps), thus each replicate contained four treatments but required five traps. The traps in the treatment with the adjacent traps were rotated by moving one of the traps of the pair to the next location in the replicate daily. An effort was made to rotate the traps for the different species relative to location within the pairs so as to randomize the possible wind directional effects of one species on the other.

Statistical analyses involving three or more treatments were made using the general linear models procedure and means were separated using Duncan's (1955) multiple-range test (SAS, 1982) with $\alpha = 0.05$. For comparing two treatments, Student's t test ($\alpha = 0.05$) was used.

RESULTS AND DISCUSSION

The results of placing BCW and BW pheromone dispensers in the same or adjacent traps are summarized in Table 2. Mean numbers of males captured in traps baited only with the BW dispensers were not significantly different from the captures of BW when adjacent traps were baited with white laminated plastic dispensers of BCW. When the same trap was baited with the dispensers of both species, there was a significant difference in captures of BW in only the three-day rotation from September 7 to 10, 1984. In contrast, significantly fewer BCW males were captured when dispensers for both species were on the same trap when compared to the numbers captured in traps baited for BCW only. Similar numbers of BCW were captured in two of three rotations when adjacent traps were baited with dispensers of BCW and BW. Although the data are not presented in tabular form, there were no significant differences in BW male captures when traps were baited only with BW dispensers ($\bar{X} \pm$ SD males/trap/night = 43.9 $\pm$ 25.4) and when the BW dispensers were placed in the same trap with white plastic laminate (41.8 + 24.8) or rubber septa (38.8 $\pm$ 25.7) BCW dispensers.

For both BW and TAW, there were significant reductions in captures of males when adjacent traps were baited with the dispensers, and virtually no males of either species were captured when the pheromone dispensers were used to bait the same trap (Table 3).

FAW rubber septa pheromone dispensers greatly reduced BW capture when both dispensers were placed in the same trap (Table 4). Although the mean number of BW males captured when adjacent traps were baited with individual dispensers was significantly reduced from that when the BW dispensers were used alone, a relatively large number of BW males were captured in the adjacent traps. Similar numbers of FAW were captured when the FAW rubber septum dispenser was used alone as when it was used in adjacent traps with BW dispensers. There was a significant reduction in captures of FAW when dispensers for both species were used to bait the same trap, although males were still caught.

When polyvial dispensers for FAW and BW laminated plastic dispensers were evaluated, there was a reduced effect on capture of BW males when dispensers of both species were used to bait the same trap (Table 5). These reductions of BW captures by the FAW dispensers when both were on the same trap are in agreement with the report of Carpenter et al. (1984). When dispensers were used in adjacent traps, however, the polyvial dispenser for FAW did not significantly influence the capture of BW males. When captures of FAW were low during the initial evaluation period (September 22–25, 1984), there were

TABLE 2. CATCHES OF BOLLWORM (BW) AND BLACK CUTWORM (BCW) IN SINGLE TRAPS SPACED 50 m APART, BAITED WITH PHEROMONE OF EITHER OR BOTH SPECIES AND IN PAIRED TRAPS IN CLOSE PROXIMITY WITH BW PHEROMONE IN ONE AND BCW PHEROMONE IN THE OTHER

	Males (mean ± SD) captured/trap/night[a]					
	BW			BCW		
Treatment	30 Aug–2 Sept	3–6 Sept	7–10 Sept	30 Aug–2 Sept	3–6 Sept	7–10 Sept
BW (alone)	28.2 ± 11.8a	35.4 ± 24.3a	37.4 ± 17.2a	0.0b	0.0c	0.1 ± 0.3b
BCW (alone)	0.3 ± 0.4b	0.4 ± 0.6b	0.1 ± 0.3c	3.7 ± 5.0a	16.8 ± 12.3a	4.3 ± 3.1a
BW and BCW (same trap)	26.4 ± 13.1a	39.6 ± 25.3a	26.8 ± 14.3b	0.1 ± 0.3b	0.1 ± 0.3c	0.0b
BW (paired)	30.4 ± 16.0a	42.7 ± 29.8a	38.4 ± 22.4a	0.0 ± 0.0b	0.1 ± 0.3c	0.0b
BCW (paired)	0.1 ± 0.4b	1.4 ± 3.0b	0.1 ± 0.3c	2.3 ± 3.6a	9.8 ± 5.7b	3.9 ± 3.8a

[a] White laminated plastic BCW dispensers were used; 1984. Means in the same column followed by different letters are significantly different according to Duncan's (1955) multiple-range test with $\alpha = 0.05$.

TABLE 3. CATCHES OF BOLLWORM (BW) AND ARMYWORM (TAW) IN SINGLE TRAPS SPACED 50 m APART, BAITED WITH PHEROMONE OF EITHER OR BOTH SPECIES AND IN PAIRED TRAPS IN CLOSE PROXIMITY BAITED WITH BW PHEROMONE IN ONE AND TAW PHEROMONE IN THE OTHER (1984)

	Males (mean ± SD) captured/trap/night[a]			
	BW		TAW	
Treatment	11–14 Sept.	15–18 Sept.	11–14 Sept.	15–18 Sept.
BW (alone)	13.2 ± 3.7a	20.6 ± 10.1a	0.0 ± 0.0c	0.0c
TAW (alone)	0.1 ± 0.3c	0.3 ± 1.0c	2.3 ± 1.9a	5.3 ± 3.0a
BW and TAW (same trap)	0.0c	0.0c	0.0c	0.1 ± 0.3c
BW (paired traps)	10.9 ± 4.5b	11.1 ± 7.4b	0.0c	0.0c
TAW (paired traps)	0.0c	0.1 ± 0.3c	0.9 ± 1.6b	2.6 ± 1.9b

[a] Means in the same column followed by different letters are significantly different according to Duncan's (1955) multiple-range test ($\alpha = 0.05$).

no significant differences in the mean captures of FAW in the three treatments. When the FAW captures increased (September 26–29, 1984), the only significant difference was between the traps baited simultaneously for FAW and BW and the FAW traps adjacent to the BW traps. A separate evaluation comparing the influence of rubber septa and polyvial pheromone dispensers for FAW on

TABLE 4. CATCHES OF BOLLWORM (BW) AND FALL ARMYWORM (FAW) IN SINGLE TRAPS SPACED 50 m APART, BAITED WITH PHEROMONE OF EITHER OR BOTH SPECIES AND IN PAIRED TRAPS IN CLOSE PROXIMITY BAITED WITH BW PHEROMONE IN ONE AND FAW PHEROMONE IN THE OTHER

	Males (mean ± SD) captured/trap/night[a]	
Treatment	BW	FAW
BW (alone)	94.2 ± 33.2a	0.1 ± 0.3c
FAW (alone)	0.1 ± 0.2c	14.1 ± 9.0a
BW and FAW (same trap)	1.1 ± 0.2c	9.0 ± 4.8b
BW (paired traps)	76.6 ± 28.0b	0.1 ± 0.3c
FAW (paired traps)	0.7 ± 0.9c	15.2 ± 7.5a

[a] Rubber septa dispensers were used for FAW; (13–21 Aug, 1984). Means in the same column followed by different letters are significantly different according to Duncan's (1955) multiple-range test ($\alpha = 0.05$).

TABLE 5. CATCHES OF BOLLWORM (BW) AND FALL ARMYWORM (FAW) IN SINGLE TRAPS SPACED 50 m APART, BAITED WITH PHEROMONE OF EITHER OR BOTH SPECIES AND IN PAIRED TRAPS IN CLOSE PROXIMITY BAITED WITH BW PHEROMONE IN ONE AND FAW PHEROMONE IN THE OTHER

	Males (mean ± SD) captured/trap/night[a]			
	BW		FAW	
Treatment	22–25 Sept	26–29 Sept	22–25 Sept	26–29 Sept
BW (alone)	46.1 ± 29.9a	136.1 ± 72.1a	0.1 ± 0.3b	0.4 ± 0.6c
FAW (alone)	0.0b	0.2 ± 0.4c	2.5 ± 3.5a	11.4 ± 15.5ab
BW and FAW (same trap)	10.5 ± 10.1b	48.0 ± 39.1b	2.3 ± 3.3a	6.4 ± 8.3b
BW (paired traps)	50.7 ± 42.0a	139.6 ± 80.6a	0.1 ± 0.3b	0.2 ± 0.4c
FAW (paired traps)	0.1 ± 0.3b	0.5 ± 0.7c	3.1 ± 3.4a	13.1 ± 17.7b

[a] Polyvial dispensers were used for FAW; 1984. Means in the same column followed by different letters are significantly different according to Duncan's (1955) multiple-range test ($\alpha = 0.05$).

capture of BW males when baiting the same trap verified that the rubber septa dispensers had a significantly greater reductive effect than the polyvial dispensers on the capture of BW males. With both types of FAW dispensers, the reduction in BW captures was significant when compared to traps baited only for BW (Table 6). These results indicate that additional components in the rubber septa dispensers for FAW have a greater effect than the one component in the polyvial dispensers for reducing male BW captures in traps baited simultaneously for both species. The effect of the laminated plastic dispenser for BW on the capture of FAW with the rubber septa and polyvial dispensers was not consistent among the three collection periods. Carpenter et al. (1984) have reported significant reductions of FAW captures with the polyvial dispenser when in combination with the BW dispensers.

Evaluation of the influence of laminated plastic dispensers for BCW and rubber septa dispensers for TAW on captures of FAW males when the dispensers were used to bait the same trap indicated that there was a significant effect of the TAW dispensers on captures of FAW only during the initial three-day period (September 18–20, 1984) (Table 7). For the other four evaluation periods, there were no significant differences between mean captures. Very few FAW males were captured when BCW dispensers were placed in the same trap with the FAW dispensers.

Captures in traps baited with TAW and BCW sex pheromones were compared with both pheromones formulated in rubber septa. There was no significant effect on BCW captures when the TAW dispensers were placed either on the same or adjacent traps (Table 8). TAW captures were extremely low when

TABLE 6. CATCHES OF BOLLWORM (BW) AND FALL ARMYWORM (FAW) IN SINGLE TRAPS SPACED 50 m APART, BAITED WITH PHEROMONE OF BW OR BOTH SPECIES

	Males (mean ± SD) captured/trap/night[a]					
	BW			FAW		
Treatment	22–24 Aug	25–27 Aug	28–30 Aug	22–24 Aug	25–27 Aug	28–30 Aug
BW (alone)	38.8 ± 21.6a	266.9 ± 117.2a	98.0 ± 47.1a	0.0b	0.4 ± 0.8b	0.0b
BW + FAW rubber septa dispensers (same trap)	0.2 ± 0.4b	4.5 ± 4.8c	0.2 ± 0.4c	4.7 ± 5.2a	6.1 ± 6.4ab	17.3 ± 7.3a
BW + FAW polyvial dispenser (same trap)	12.1 ± 12.2b	93.1 ± 49.7b	29.4 ± 14.4b	1.3 ± 1.4b	11.1 ± 10.0a	2.1 ± 2.0b

[a]Rubber septa and polyvial dispensers for FAW were used; 1984. Means in the same column followed by different letters are significantly different according to Duncan's (1955) multiple-range test with $\alpha = 0.05$.

TABLE 7. CATCHES OF FALL ARMYWORM (FAW) IN WIDELY SPACED SINGLE TRAPS BAITED WITH PHEROMONE OF FAW ALONE OR IN COMBINATION WITH PHEROMONE OF ARMYWORM (TAW) OR BLACK CUTWORM (BCW)[a]

Treatment	FAW males (mean ± SD) captured/trap/night[b]				
	18–20 Sept	21–23 Sept	24–26 Sept	27–29 Sept	30 Sept–1 Oct
FAW (alone)	72.1 ± 35.1a	49.7 ± 117.2a	32.7 ± 15.2a	13.1 ± 11.9a	4.4 ± 4.5ab
FAW + TAW (same trap)	51.3 ± 33.2b	49.6 ± 57.9a	26.2 ± 19.2a	10.4 ± 8.5a	5.4 ± 5.1a
FAW + BCW (same trap)	4.8 ± 4.9c	4.2 ± 2.9b	0.4 ± 0.9b	1.0 ± 1.3b	1.9 ± 2.2b

[a]Laminated plastic dispensers were used for BCW and rubber septa dispensers for both TAW and FAW; 1984. Test run in a field of sweet corn with two trap lines 100 m apart, one line of six traps and one line of three traps, traps within lines 42 m apart.
[b]Means in the same column followed by different letters are significantly different according to Duncan's (1955) multiple-range test with $\alpha = 0.05$.

both BCW and TAW dispensers were placed on the same trap; however, there was no significant effect when the dispensers for the two species were placed on adjacent traps.

No FAW males were captured when FAW and TAW rubber septa dispensers were placed on the same trap (Table 9). When the dispensers of the two species were placed on adjacent traps, there was significant but not complete reduction in the captures of TAW males.

The results obtained clearly indicate that multispecies trapping involving combinations of the sex pheromone dispensers for BW, BCW, TAW, and FAW

TABLE 8. CATCHES OF ARMYWORM (TAW) AND BLACK CUTWORM (BCW) IN SINGLE TRAPS SPACED 50 m APART, BAITED WITH PHEROMONE OF EITHER OR BOTH SPECIES AND IN PAIRED TRAPS IN CLOSE PROXIMITY BAITED WITH TAW PHEROMONE IN ONE AND BCW PHEROMONE IN THE OTHER

Treatment	Males (mean ± SD)/trap night[a]	
	BCW	TAW
BCW (alone)	11.9 ± 18.8a	0.1 ± 0.3b
TAW (alone)	0.0b	16.8 ± 13.8a
BCW and TAW (same trap)	14.0 ± 21.1a	0.1 ± 0.3b
BCW (paired traps)	14.0 ± 27.2a	0.0b
TAW (paired traps)	0.1 ± 0.3b	18.9 ± 14.0a

[a]Rubber septa dispensers were used for both species; 30 Sept–3 Oct, 1987. Means in the same column followed by different letters are significantly different according to Duncan's (1955) multiple-range test ($\alpha = 0.05$).

TABLE 9. CATCHES OF ARMYWORM (TAW) IN SINGLE TRAPS SPACED 50 m APART, BAITED WITH PHEROMONE OF EITHER TAW ALONE OR BOTH TAW AND FALL ARMYWORM (FAW) AND IN PAIRED TRAPS IN CLOSE PROXIMITY WITH TAW PHEROMONE IN ONE AND FAW PHEROMONE IN THE OTHER (1987)[a]

Treatment	Males (mean ± SD)/trap/night[b]		
	25–26 Mar	27–28 Mar	29 Mar and 1 Apr
TAW (alone)	69.3 ± 16.7a	30.8 ± 11.7a	19.2 ± 4.1a
TAW and FAW (same trap)	0.0b	0.2 ± 0.4b	0.0b
	8–9 Apr	10–11 Apr	8–11 Apr
TAW (alone)	20.3 ± 4.5a	22.0 ± 6.1a	21.2 ± 5.2a
TAW and FAW (paired traps)	2.5 ± 1.5b	2.3 ± 2.1b	2.4 ± 1.7b

[a] Replicated three times.
[b] Means for the different periods in the same column followed by different letters are significantly different according to Student's t test ($\alpha = 0.05$).

in the same trap results in reduced catches. Although captures of one species were not influenced in some of the combinations evaluated, captures of at least one of the species was always affected, thus precluding use of any of the combinations. The results also demonstrated the species specificity of the sex pheromone identified for the different species. It is difficult to speculate which pheromone components were responsible for the incompatibility of pheromone dispensers for the different species because of differences in components and in their ratios. From a mating disruption standpoint, it would be important to determine which specific components contributed to the incompatibility. The interaction between captures of these species when adjacent traps were baited with sex pheromone dispensers of two different species indicates that spacing between pheromone traps is critical. Therefore, spacing of traps used to monitor these species at the same site should be studied in further detail.

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TRAIL PHEROMONE OF THE ANT *Tetramorium impurum* AND MODEL COMPOUNDS: STRUCTURE–ACTIVITY COMPARISONS[1]

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Abstract—The major component of the trail pheromone of *T. impurum* is methyl 2-hydroxy-6-methylbenzoate (methyl 6-methyl salicylate). The poison reservoir of each worker contains about 1.0 ng of this substance in the venom. To ascertain the degree of specificity of the substance as pheromone and to determine any correlation between structure and biological activity, 38 synthetic analogs were tested in a bioassay. Sixteen compounds showed activity at 100 ng per artificial trail. The importance of the methyl group and methyl ester were demonstrated, while the hydroxyl group has a relatively small effect.

Key Words—*Tetramorium impurum*, Hymenoptera, Formicidae, trail pheromone, ant, venom, methyl 6-methylsalicylate, structure–activity correlation.

INTRODUCTION

Tetramorium impurum Foerster and *T. caespitum* L. are morphologically almost indistinguishable and said to be differentiated only by the male genitalia (B. Poldi, quoted in Pasteels et al., 1981). Chemically they are more easily distinguished by the contents of their Dufour glands (Billen et al., 1986), but a still more striking difference is that they do not use the same trail pheromone. Available information indicates that some trail pheromones are shared by many spe-

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cies of ant (Attygalle and Morgan, 1985), and the phenomenon of shared trail pheromones is not confined to ants (Morgan, 1990a). For example, 3-ethyl-2,5-dimethylpyrazine (EDMP) is used by all 13 species of *Myrmica* so far examined and also by a number of other species in other genera (Jackson et al., 1989).

The trail pheromone of *T. caespitum* is a 7:3 mixture of 2,5-dimethylpyrazine and EDMP (Attygalle and Morgan, 1983). We already have reported briefly the isolation of the trail pheromone of *T. impurum* and its identification as methyl 6-methylsalicylate (Morgan and Ollett, 1987). We now report the experimental evidence for the isolation and identification of this substance together with quantitative information on the extent of trail following evoked by a gland extract and the pure compound in a simple bioassay. Because of the simplicity of the compound, and its essentially two-dimensional shape, a number of simple analogs and derivatives were obtained or prepared to draw some conclusions about the necessary and sufficient structural features required for activity as this pheromone.

METHODS AND MATERIALS

T. impurum colonies were collected at various site in Belgium and France by R. and M.C. Cammaerts and sent live by post to Keele, where they were maintained in artificial nests in plastic bowls, lined with Fluon emulsion (ICI, Blackley) and fed on sugar water and dipteran larvae.

Bioassays were performed using the method of Pasteels and Verhaeghe (1974) in which a circle (radius 5 cm) was drawn on white paper and the circumference marked off into 1-cm arcs. The solution or extract to be tested was placed in a Standardgraph funnel pen (Blundell Harling, Weymouth) and the solution placed on the circumference of the circle. After 2 min to allow the solvent to evaporate, the sheet of paper was placed in the foraging area of the ant colony. Individuals walking onto the paper were observed for a period of 20 min. Ants approaching the circle either walked across it without detecting the odor (scored zero) or turned and followed the circumference for a time, when the number of centimeter arcs of trail followed without deviation were counted and their medians recorded in the tables; ants that ran a continuous path of 50 cm then were removed from the test. Each experimental solution was preceded by a control experiment in which the pen was filled with hexane alone, which was placed on the circle and observed in the same way. The pen was flushed thoroughly with hexane between each test solution.

Extracts of poison reservoirs or other glands in hexane were made in a small tissue grinder made from a glass rod and a small test tube roughened with carborundum paste. Solutions of pure chemicals of known concentration were prepared in hexane and diluted to the required concentration. All compounds

were examined by gas chromatography–mass spectrometry (GC-MS) to determine their purity. The compounds were all tested at 100 ng per trail initially and, if found active, were tested at 10-fold dilutions until activity ceased.

For thin-layer chromatography (TLC), a hexane extract of two poison glands was placed at the origin of a silica TLC plate (5 × 20 cm), which was then developed with hexane–acetone (3 : 2). The plate was dried and the silica cut into 10 equal bands, according to the R_f regions 0 to 1.0. The silica from each band was placed in a glass tube, plugged with glass wool, and any active compounds were eluted with acetone and the eluant tested in the bioassay.

Gas chromatography with trapping of the effluent was carried out as described by Evershed et al. (1982) using the splitter and trapping apparatus illustrated by Attygalle and Morgan (1988), with a packed column coated with OV-101, temperature program from 50 to 250°C at 10°C/min, and using the solventless solid injection method of Morgan and Wadhams (1972; see also Morgan, 1990b).

Identification of the pheromone compound was made by GC-MS. The poison glands of 10 workers were sealed in a glass capillary, which was introduced into the gas chromatograph by the method of Morgan and Wadhams (1972). After heating for 2 min to 140°C, the capillary was crushed and the volatile materials were flushed onto the chromatography column in a Hewlett Packard 5890 GC linked to a 5970B Mass Selective Detector with a HP 59970C ChemStation data processor. A fused silica capillary column (12 m × 0.2 mm) coated with HP-1 (cross-linked methylsilicone gum, equivalent to OV-1) of 0.33 μm film thickness was connected through a length of deactivated silica tubing (10 m × 0.32 mm) to the source of the mass spectrometer. The carrier gas was helium at 10 psi column head pressure. Injection was made in the splitless mode, and the injection port purge was turned on 30 sec after the injection. The oven temperature was initially at 30°C for 2 min, then increased to 250°C at 8°C/min. The Mass Selective Detector was set to monitor m/z 35–350 in the scan mode (~ 1.5 scans per second) under Autotune conditions using 70 eV ionization.

The quantity of methyl 6-methylsalicylate in the glands was determined by measuring the peak area obtained above and comparing it with the peak areas obtained by injecting known amounts of the synthetic compound at similar levels of dilution.

Twenty-six methyl esters were prepared from the corresponding acids by treatment with diazomethane in ether. Ethyl 6-methylsalicylate was prepared by bromination–dehydrobromination of ethyl 6-methyl-2-oxocyclohex-3-enecarboxylate, itself prepared by alkaline condensation of ethyl acetoacetate and crotonaldehyde by the method of Hauser and Pogany (1980). 2-Methoxy-6-methylbenzoic acid was prepared by the method of Anslow and Raistrick (1931), and then esterified with diazomethane. A number of aminoacids were the gift

of G.J. Thomas, others were gifts of C.J.W. Brooks or were available commercially. The purity of all synthetic compounds was checked by GC-MS.

RESULTS

Extracts made from the poison gland, Dufour gland, abdomen without the poison apparatus, head, and legs of *T. impurum* workers were all tested in the bioassay. Only the poison gland showed activity. The Dufour gland and other body parts were inactive. One poison gland had median activity of 8 cm in the bioassay, 0.1 poison glands had activity of 9.5 cm, 0.01 glands had activity of 3 cm, and at 0.001 glands, no activity was detectable. This test depended upon the age of the colony, and when the colony had been cultured in the laboratory for one year, the ability of workers to follow a glandular extract or pure methyl 6-methylsalicylate fell to zero. The test of ability of workers to follow an extract of one Dufour gland of a worker therefore was checked at frequent intervals, and most of the tests were carried out within four months of collection of the colony.

After thin-layer chromatography of a hexane extract of two poison glands, the band of R_f 0.6–0.7 showed strong activity in bioassay (median of 18 cm) while other R_f bands were inactive. Reaction of the gland extract with bromine, diazomethane, or conc. hydrochloric acid before chromatography had no effect on R_f value or activity.

Gas chromatography of 10 glands by the solid sampling method (Morgan and Wadhams, 1972), collecting three broad bands, gave one band eluting between 5 and 15 min, strongly active in bioassay (median value 22 cm). Repeating the experiment, but collecting the effluent in 1.5-min intervals gave only one strongly active band (Table 1), corresponding to a Kovats index between 1300 and 1400.

When 10 poison glands were subjected to coupled GC-MS, the only peak visible in the 1300–1400 Kovats index range was identified by its mass spectrum as either methyl 6-methylsalicylate or methyl 5-methylsalicylate [M^+ 166 (27%), *m/z* 134 (100), 106 (56), 105 (49), 78 (45), 77 (38), 63 (9), 51 (24)]. Both compounds gave the same mass spectrum but had different retention times. The mass spectrum and retention time corresponded to those of a synthetic sample of methyl 6-methylsalicylate. The synthetic compound had R_f 0.68 in the TLC system used and had a retention time of 6.5 min on the OV-101 column. In bioassay the synthetic methyl 6-methylsalicylate gave median values of 21 cm at 10 ng/trail and 5 cm at 1 ng/trail (Table 2). Methyl 5-methylsalicylate was inactive (Table 2).

By comparison of areas of the methyl 6-methylsalicylate peak from 10 poison glands with peak areas from similar known amounts of synthetic com-

TABLE 1. ACTIVITY OF VOLATILE FRACTIONS OF CONTENTS OF 10 POISON GLANDS OF *T. impurum*, SUBJECTED TO GAS CHROMATOGRAPHY, AND COLLECTING 95% OF MATERIAL FOR BIOASSAY[a]

Fraction	Elution time interval (min)	Activity (median cm)
1	4.5–6.0	5
2	6.0–7.5	19
3	7.5–9.0	3
4	9.0–10.5	0
5	10.5–12.0	0
6	12.0–13.5	1
7	13.5–15.0	0

[a] For column and temperature program, see Methods and Materials.

pounds, it was calculated that the mean amount per worker poison gland was 1.0 ng.

The activity in bioassay of all the synthetic compounds tested are listed in Table 2. The Wilcoxon-Mann-Whitney test (Siegel and Castellan, 1988) was applied to the results of the bioassays. All nonzero medians were significantly different ($P < 0.001$) from the controls.

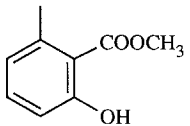
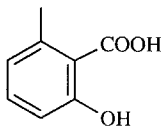
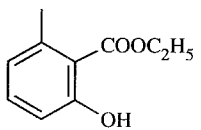
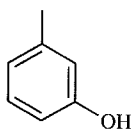
DISCUSSION

Preliminary experiments showed that the trail pheromone of *T. impurum* was contained in the venom of workers. TLC and GC experiments indicated that only one substance or, if more than one, a group of substances very closely related in polarity and mass was responsible for the trail-following behavior of workers. The only detectable peak in the active region of the gas chromatogram was methyl 6-methylsalicylate, and this compound was highly active in the bioassay. No dimethylpyrazine or ethyldimethylpyrazine was present in *T. impurum* venom, even at $\frac{1}{10}$ the amount present in *T. caespitum*. Similarly, *T. caespitum* venom glands provided no evidence for the presence of methyl 6-methylsalicylate. The later compound had no effect in inducing trail-following in *T. caespitum*.

The average amount of methyl 6-methylsalicylate per worker venom gland (1.0 ng) was the same as that reported in our preliminary communication (1 ng) (Morgan and Ollett, 1987). The two determininations were performed two years apart by different operators on different colonies from different locations.

Methyl 6-methylsalicylate has been identified in insect exocrine glands a

TABLE 2. ACTIVITY OF COMPOUNDS MEASURED BY MEDIAN NUMBER OF 1-cm ARCS RUN WITHOUT INTERRUPTION ON CIRCULAR TRAIL OF RADIUS 5 cm[a]

Name	Structure	Concentration (ng/trail)				
		100	10	1	0.1	0.01
1 Methyl 6-methylsalicylate		4(50+)	21(50+)	5(50+)	1(9)a	
2 6-Methylsalicylic acid		0(4)				
3 Ethyl 6-methylsalicylate		3(14)	0(2)			
4 *m*-Cresol		3(50+)a	0(29)a			

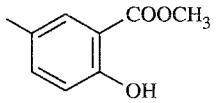
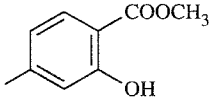
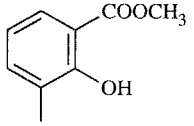
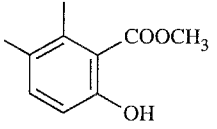
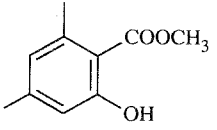
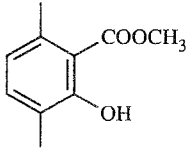
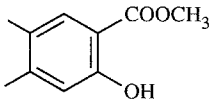
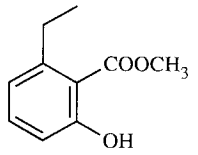
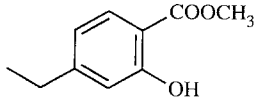
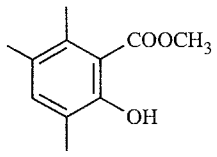
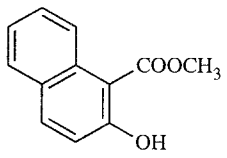
5 Methyl 5-methylsalicylate		0(44)	0(2)			
6 Methyl 4-methylsalicylate		0(25)	0(2)			
7 Methyl 3-methylsalicylate		0(13)	0(2)			
8 Methyl 5,6-dimethylsalicylate		5(46)	0(6)			
9 Methyl 4,6-dimethylsalicylate		2(50+)	4(50+)a	3(50+)b	4(50+)b	2(50+)a[b]
10 Methyl 3,6-dimethylsalicylate		3(50+)a	4(45+)b	7(50+)b	4(50+)b	2(50+)a[b]

TABLE 2. Continued

Name	Structure	Concentration (ng/trail) 100	10	1	0.1	0.01
11 Methyl 4,5-dimethylsalicylate		0(5)	0(2)			
12 Methyl 6-ethylsalicylate		0[c]				
13 Methyl 4-ethylsalicylate		0[c]				
14 Methyl 3,5,6-trimethylsalicylate		0(2)a				
15 Methyl 2-hydroxy-1-naphthoate		0(10)				

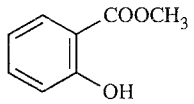
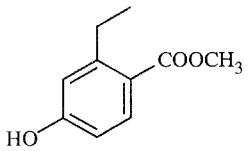
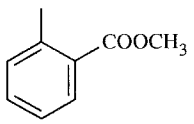
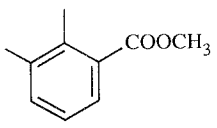
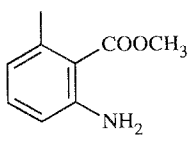
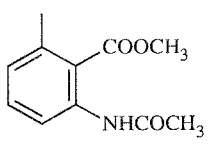
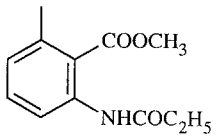
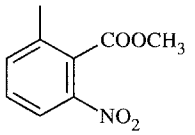
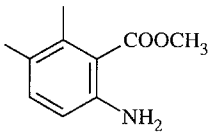
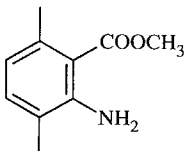
16 Methyl salicylate		1(50+)	0(1)		
17 Methyl 2-ethyl-4-hydroxybenzoate		0[c]	0[c]		
18 Methyl *o*-toluate		9(50+)	3(36)	0(5)	
19 Methyl 2,3-dimethylbenzoate		0(16)	0(4)		
20 Methyl 2-amino-6-methylbenzoate		7(48)	0(4)		
21 Methyl N-acetyl-2-amino-6-methylbenzoate		3(34)	5(50+)	3(50+)a	0(39)

TABLE 2. Continued

Name	Structure	Concentration (ng/trail) 100	10	1	0.1	0.01
22 Methyl N-propionyl-2-amino-6-methylbenzoate		0(50+)	0(1)			
23 Methyl 6-methyl-2-nitrobenzoate		0(2)				
24 Methyl 2-amino-5,6-dimethylbenzoate		0(3)				
25 Methyl 2-amino-3,6-dimethylbenzoate		0(10)				

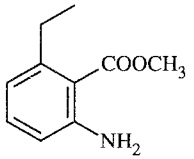
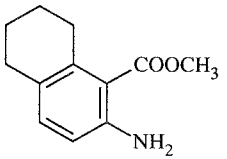
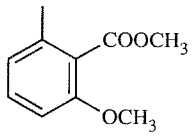
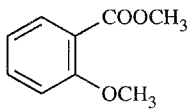
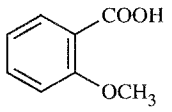
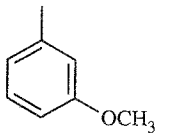
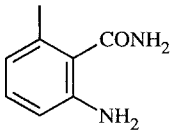
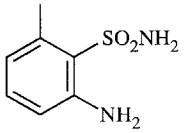
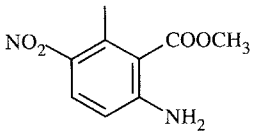
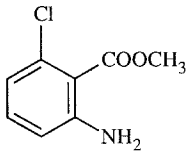
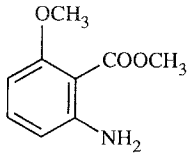
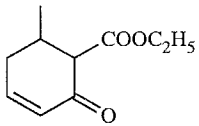
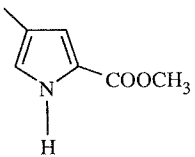
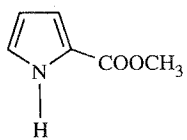
26 Methyl 2-amino-6-ethylbenzoate		20(50+)a	22(50+)	0(8)
27 Methyl 2-amino-5,6,7,8-tetrahydro-1-naphthoate		0(24)	0(1)	
28 Methyl 2-methoxy-6-methylbenzoate		2(12)	1(8)	0(5)
29 Methyl *o*-anisoate		4(29)a	1(50+)	1(34)
30 *o*-Anisic acid		3(50+)	0(18)	
31 *m*-Methoxytoluene		1(42)a	0(17)b	

TABLE 2. Continued

Name	Structure	Concentration (ng/trail) 100	10	1	0.1	0.01
32 2-Amino-6-methylbenzamide		0(5)				
33 2-Amino-6-methylbenzenesulfonamide		16(31)	7(32)	1(8)		
34 Methyl 6-amino-2-methyl-3-nitrobenzoate		0(50+)	0(2)			
35 Methyl 2-amino-6-chlorobenzoate		0(26)	0(2)			

36 Methyl 2-amino-6-methoxybenzoate		0(25)	0(2)
37 Ethyl 6-methyl-2-oxocyclohex-3-enecarboxylate		0(3)	
38 Methyl 4-methylpyrrole-2-carboxylate		0(18)	0(3)a
39 Methyl pyrrole-2-carboxylate		0(15)	

[a] According to the test of Pasteels and Verhaeghe (1974). All compounds were tested at 100 ng/trail and if active, tested at 10-fold dilutions until activity ceased. All tests were preceeded by a control test with hexane only which showed no activity (median = 0). The lower extreme value was zero for all tests, the upper extreme values are given in parentheses after each median; 50+ indicates that the ant followed for 50 continuous arcs, after that the ant was removed from the test. Workers were observed for 20 min, during that time between 30 and 135 observations were made, except (a) where 21–30 counts were made and (b) where 16–20 counts were made. All nonzero medians were significantly different from the control (Wilcoxon-Mann-Whitney test, $P < 0.001$).

[b] Activity was not reproducible at lower concentrations.

[c] Median values only recorded.

number of times before, but this is the first time it has been found in the venom of an ant or identified as a trail pheromone. It is also the first simple benzenoid compound found to serve as a trail pheromone. The compound is found in the mandibular glands of males of a number of *Camponotus* species, where it has been associated with the swarming of males and virgin females (Lloyd et al., 1984). For a summary of its occurrence also in ponerine ant workers' mandibular glands and the pygidial defensive secretion of a beetle, see Lloyd et al. (1984).

The activity of dimethylsalicylates (compounds 9 and 10, Table 2) in the bioassay at high dilution, higher than the identified natural pheromone was surprising, and caused us to look carefully to see if small amounts of such a compound were present in the poison reservoir. Using selected ion monitoring, a related compound with longer retention time and 14 units greater mass was discovered in the venom, at approximately 50 pg/insect. However, neither the retention time nor the mass spectrum corresponded to a methyl dimethylsalicylate. We expected, therefore, that methyl 6-ethylsalicylate, already identified in insects (Gnanasunderam et al., 1984), and highly probable on biosynthetic grounds, would be the substance. Methyl 6-ethylsalicylate was synthesized from 2-pentenal and methyl acetoactate by the general method of Hauser and Pogany (1980); however, the synthetic compound did not show much activity in the bioassay and possessed a different mass spectrum. The naturally occurring minor component was finally identified as methyl 2-methoxy-6-methyl benzoate by comparison with a synthetic specimen. The spectra of methyl 6-ethylsalicylate and methyl 2-methoxy-6-methylbenzoate are shown in Figure 1, since these compounds are likely to be confused. Compounds of this group with a free hydroxyl group show a strong M^+-32 ion in their mass spectra, due to loss of methanol. The methyl ether shows a strong M^+-31 ion through loss of a methoxy radical.

Model compounds. The effectiveness in the bioassay of a number of compounds of structure similar to methyl 6-methylsalicylate is given in Table 2. Each compound was tested initially at 100 ng/trail. At this concentration, workers show by their behavior that they can detect the compound, even if it is too concentrated for them to follow it well. If active, the compound was tested at successive 10-fold dilutions. Eleven compounds were detected by the foraging ants at 100 ng/trail, and some ants followed the artificial trail, although the median values were zero. These substances were tested again at 10 ng/trail. None were active.

That most of the compounds were inactive attests to the specificity of structure required for the antennal receptors to induce trail-following. The small number of model compounds that showed activity is still more interesting. Compounds 2–4 (Table 2) show the importance of the methyl ester. Conversion to the free acid, or to its ethyl ester, or the total absence of the group destroys

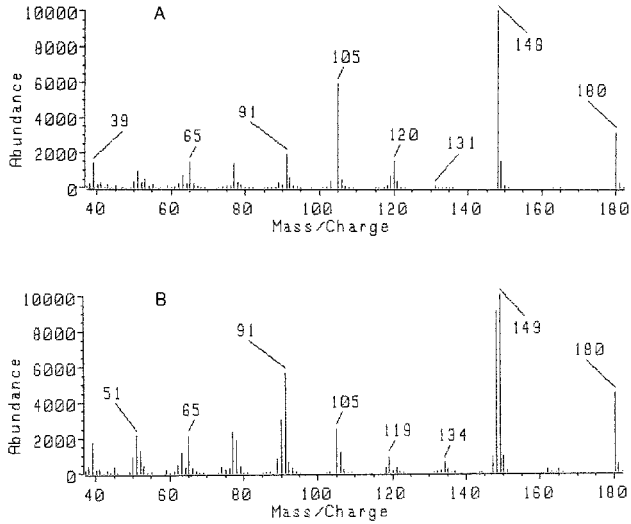


FIG. 1. Mass spectra of methyl 6-ethylsalicylate (A) and methyl 2-methoxy-6-methylbenzoate (B). The differences in the mass spectra, particularly the relative intensities at *m/z* 149, 120, 105, 91, and 78, are easily apparent. There was no evidence of (A) in *T. impurum* poison glands but (B) was identified in picogram quantities by selective ion monitoring.

activity. Compounds 5–15 explore the activity of the methyl group. Movement of the group to another position (compounds 5–7) or its total absence (16) completely removes activity, but an extra methyl group in addition to that at C-6 (compounds 8–10) gave activity at higher dilution beyond that of compound 1, but a third methyl (14) seems to destroy it again. 6-Ethylsalicylates are known in nature and several were tried (12, 13, 17), but they did not show high activity (but see 26). Another aromatic ring fused in the 5,6 positions (15) destroyed activity. Compounds 17–28 explored the effect of the hydroxyl group. Here the evidence is less clear, but the OH appears to be a less critical part for activity, for its total absence (18) does not destroy activity completely, but the loss of activity in the dimethyl benzoate (19) is confusing. Replacement of OH by NH_2 (20) still shows some activity, and the acetylation of this (21) enhanced its activity. Lengthening the chain by one carbon atom to an *N*-propionyl amide (22) again destroyed activity. Not surprisingly, replacing OH by the strongly electron-withdrawing nitrogroup gave an inactive compound (22). Since compound 20, in which NH_2 replaces OH, shows some activity, 25, by analogy with 10, might be expected to be active but was not. Compounds 24, 26, and 27 are further variations in this series.

The methyl ether of the parent pheromone, methyl 2-methoxy-6-methyl-

benzoate (28), present in the gland in picogram quantities, was also active but less than so its parent. It showed some slight synergistic effect when mixed with methyl 6-methylsalicylate, but this was difficult to quantify because the colony was aging when this test was made. Other methoxy derivatives (29–31 and 36) showed expected behavior, i.e., only methyl *o*-anisoate (29) was appreciably active. Methyl 6-methoxysalicylate was prepared too late to test. The group 32–36 are miscellaneous related structures. The comparatively high activity of the aminosulfonamide (32) was surprising, but recalls the way sulfanilamide (parent of the sulfa drugs) mimics the structure of *p*-aminobenzoate in inhibiting the synthesis of folic acid bacteria (Sexton, 1963). It is tempting to suggest that this substance is strongly adsorbed on the antennal receptors. Compound 37, an intermediate in the synthesis of 3, is, in its enol form, a dihydroderivative of 3. It was therefore interesting to examine it, but it had no activity. The superficial structural similarity between 1 and 38, the trail pheromone of *Atta texana* (Tumlinson et al., 1972) and *Atta cephalotes* (Riley et al., 1974) were the justification of including 38 and 39, but no activity was found.

Sonnet and Moser (1972, 1973) carried out similar structure–activity studies with *Atta texana* and compounds similar to methyl 4-methylpyrrole-2-carboxylate (37). Their work showed that some analogs of 38, with similar structure, retained activity for *Atta texana*, but too great a divergence from the true pheromone quickly destroyed it, while positional isomers of 38 were generally inactive.

The biosynthetic origin of methyl 6-methylsalicylate in the venom is unknown. The biosynthesis of 6-methylsalicylic acid from acetate-malonate has been studied in detail in microorganisms (Weiss and Edwards, 1980), and it has been reported to be synthesized in barley (Kannangara et al., 1971). In general, higher animals are not known to synthesize simple aromatic compounds, but Tecle et al. (1986) have shown positively that 2-hydroxy-6-methylacetophenone is biosynthesized from acetate via a C_{10} polyketide in a ponerine ant. Methyl 6-methylsalicylate could arise directly from this by a Baeyer–Villiger oxidation. On the other hand, mellein (3,4-dihydro-9-hydroxy-3-methylisocoumarin), normally regarded as a fungal metabolite, has been identified as the main component of the male wing gland pheromone of the bumble-bee wax moth *Aphomia sociella*, and *Aspergillus ochraceus*, a mellein-producing microorganism was detected in the gut of *A. sociella* larvae and bumble-bee nests (Kunesch et al. 1987). The biosynthetic relationship between mellein and 6-methylsalicylate and other compounds is discussed by Brand et al. (1973), Gnanasunderam et al. (1984), and Tecle et al. (1986). Brand et al. (1973) found methyl 6-methylsalicylate and mellein together in the mandibular gland secretion of males of three species of *Camponotus* ants (Subfamily Formicinae). It would have been interesting to test 2-hydroxy-6-methylacetophenone among our model compounds but it was not available in time.

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MALE PHEROMONE OF SWIFT MOTH, *Hepialus hecta* L. (LEPIDOPTERA: HEPIALIDAE)

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Abstract—(*R*)-6-Ethyl-2-methyl-2,3-dihydro-4H-pyran-4-one, (1*R*,3*S*,5*R*)-3-ethyl-1,8-dimethyl-2,9-dioxabicyclo[3.3.1]non-7-ene, and (1*R*,3*S*,5*R*)-3-ethyl-1,8-dimethyl-2,9-dioxabicyclo[3.3.1]non-7-en-6-one represent the main components in the male pheromone of the swift moth, *Hepialus hecta*. The amounts of the three components were 40, 5, and 5 μg per male, respectively. Structure elucidation of the compounds was based on spectroscopic data as compared to synthetic reference samples. The absolute configurations were determined by gas chromatography on chiral stationary phases; optically active samples served as reference compounds. Electrophysiological and behavioral experiments with natural material and synthetic samples clearly showed the three heterocyclic compounds to act as pheromones. (*E*,*E*)-α-Farnesene represents the main component of the scent secretion of male *Hepialus humuli*.

Key Words—*Hepialus*, Lepidoptera, Hepialidae, swift moth, pheromone, dihydropyrone, 2,9-dioxabicyclo[3.3.1] nonane, behavior, electrophysiology.

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INTRODUCTION

Swift moths—Hepialidae—are a primitive group of Lepidoptera. Males of a number of species in this family possess conspicuous tibial scent organs on their hind legs. In *H. hecta*, a common species in middle Europe, these organs and their impressive fruity odor have been known since the last century (Bertkau, 1882). The tibiae of the hind legs are enlarged, pear-shaped, and bear a great number of scent scales, while the tarsus is reduced and without function. During their striking pendular flights, the males erect these scales and emit the odor, which for a long time was believed to attract the females (for a review of the biology of *Hepialus* see Mallet, 1984), but until now, no behavioral or electrophysiological proof of the function of this secretion as a male sexual attractant has been reported.

The elements of male androconial scent organs in the Lepidoptera (Boppré, 1984; Boppré and Schneider, 1989) are the hair-forming trichogen cells, which in this case are glandular cells as well. The secretory products then are transmitted on the large surface of the scent scales from which the odor is released.

We now report on structure elucidation and synthesis of the volatile components that are emitted form the scent organ. In addition, we report on the essence of electrophysiological and behavioral activity, which proves the function of the identified components as pheromones. Details of these biological functions will be published elsewhere.

We also describe the identification of volatile components of similar glands of *Hepialus humuli*, a larger European relative of *H. hecta*, showing similar sexual behavior.

METHODS AND MATERIALS

Biological Material. Males of *H. hecta* (270) and *H. humuli* (50) were collected during their calling flights at locations in Bavaria, Germany during 1983 and 1984. The hindlegs were removed, immediately stored under *n*-pentane and kept at −18°C until work-up. In addition, some samples of *H. hecta* were stored in vials without solvent, and the liquid secretion was collected with a glass capillary inserted directly into the tibia subsequently analyzed by gas chromatography.

Gas Chromatography. Extracts and crude secretions were analyzed by GC on a Carlo Erba Fractovap 2101 AC gas chromatograph equipped with an FID. A 50-m CP-Sil-8-CB fused silica column (0.32 mm ID, 0.25 μm film thickness) was used, which was programmed from 60°C to 280°C at 3°C/min. Preparative gas chromatography was performed as described earlier (Sinnwell et al., 1985).

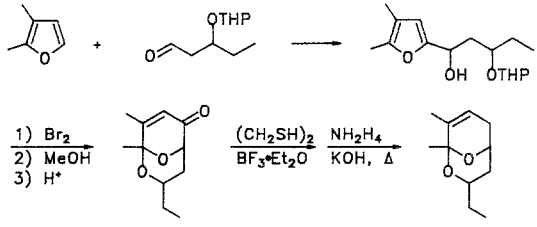
Gas chromatographic separations on optically active stationary phases were

performed on fused silica capillaries coated with XE-60-L-valine-(*S*)-α-phenylethylamide (PEA) (50 m, 0.25 mm ID, programmed from 100°C at 1.5°C/min) (König et al., 1981) or with Ni(II)-bis(heptafluorobutanoyl-(1*R*,5*S*)-pinan-4-onate) in OV-1 (50 m, 0.25 mm ID, 80°C isothermal) (Schurig and Wistuba, 1984).

Mass Spectrometry. For mass spectrometric investigation, a GC-MS system consisting of a Carlo Erba Fractovap 2100 gas chromatograph coupled to a Varian MAT 311 A mass spectrometer was used, which was equipped with the same fused silica column as described above. GC operating conditions were identical.

Synthesis. Racemic 2,6-dialkyl-2,3-dihydro-4H-pyran-4-ones **1**, **2**, and **3** (Figures 1 and 5 below) were prepared by acylation of β-ketoesters with α,β-unsaturated acylhalides followed by cyclization, saponification, and decarboxylation as described in the literature (Gelin and Gelin, 1968). Enantiomerically pure (*R*)- and (*S*)-6-ethyl-2-methyl-2,3-dihydro-4H-pyran-4-one was prepared as reported by Mori and Kisida (1986).

Racemic (1*R**,3*S**,5*R**)-3-ethyl-1,8-dimethyl-2,9-dioxabicyclo[3.3.1]non-7-en-9-one 4 was synthesized according to the strategy used by DeShong et al. (1985) for the synthesis of tirandamycine analogs (Scheme 1). 2,3-Dimethylfuran (Rice and Dyer, 1975) was metallated with *n*-butyl lithium and treated with 3-(2-tetrahydropyranyloxy) pentanal at −78°C. The resulting crude trisubstituted furan was oxidized with bromine and treated with methanol to yield the corresponding bisacetal, which upon acidic treatment yielded ketone **4** in 20% yield. The product was purified by distillation (bp 112–125°C) and column chromatography (silica gel, methylenechloride–diethyl ether 6:1). Thioacetalization of **4** was accomplished by reaction with 1,3-propanedithiol in the presence of boron trifluoride etherate. The crude thioacetal was desulfurized to yield (1*R**,3*S**,5*R**)-3-ethyl-1,8-dimethyl-2,9-dioxabicyclo[3.3.1]non-7-ene, **7**, by heating with potassium hydroxide and hydrazine in triethylenglycol (Georgian et al., 1959). Desulfurization with Raney-Ni produced only mixtures of **7** and the corresponding saturated acetal. Pure **7** was isolated by preparative gas chromatography.



SCHEME 1. Synthesis of the bicyclic acetals **4** and **7**.

Pure enantiomers of **4** and **7** were prepared as described previously (Mori and Kisida, 1986). Microreactions of crude extracts or isolated compounds used for structure elucidation were performed according to Francke et al., (1989).

Behavioral Experiments. Investigations were performed in the natural habitat, semiwet meadows, during 1984 until 1988 in southern Germany. In June and July, mating behavior, lasting about half an hour at the time around sunset, could be observed. Artificial scent sources consisted of 1 mg of the pure racemic main component **1** on cotton plugs, which were suspended on plant branches. For comparison, freshly cut scent legs and extracts thereof were used. In control experiments, untreated cotton plugs were exposed. The behavior of males and females was observed and recorded. When testing the reactions of females to the exposed scent sources, all free males were removed from the test site prior to the experiments. Full details of the behavior will be published elsewhere.

Electrophysiology. Electroantennograms and single-cell recordings were performed directly after capture of the animals on the same day. Because of the short, thick antennae and the small number and size of the sensillae, electrophysiological measurements proved to be difficult. To carry out the recording, the animal was tethered and its antennae fixed with tungsten wire. For antennogram recordings, electrodes filled with hemolymph Ringer solution (Kaissling and Thorson, 1980) were inserted into the tip and the base of the antenna or attached by means of a drop of dialyzed electrode paste (electrode cream of ECG, Hellige). An identical electrode inserted into the tip of the antenna was used as reference for the single-cell recordings. The other glass microelectrode was filled with receptor lymph Ringer and inserted into the base of single sensillum. Stimulation was performed with 1-sec air puffs through glass cartridges with odor sources (scent legs or 1 cm^2 filter paper with the odorants) and directed onto the antennae.

RESULTS

Chemical Analysis. Comparison between extracts of the whole scent leg of *H. hecta* and pure liquid secretions revealed no difference in composition except for additional common fatty acids in extracts of the scent leg. For further investigations, scent leg extracts were used for convenience. A gas chromatogram of an extract is shown in Figure 1. The main components **1**, **2**, **4**, and **7** were identified by isolation, chemical microreactions, mass spectrometry, and [^{1}H]- and [^{13}C]NMR analysis as described earlier (Francke et al., 1985; Sinnwell et al., 1985). Racemic reference samples of compounds **1**, **2**, **4**, and **7** showed the same gas chromatographic retention times and the same mass and NMR spectra as the natural compounds. Structure elucidation of the minor components **5**, **6**, **8**, and **9** were based on the results of microreactions and their

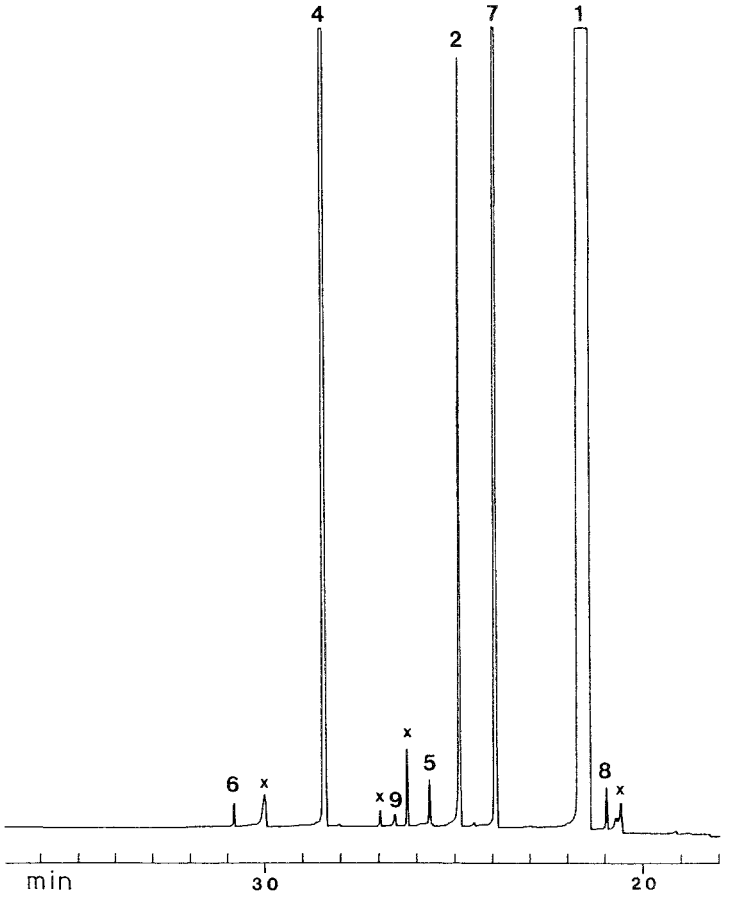


FIG. 1. Gas chromatogram of scent gland secretion of *H. hecta* (numbers according to the text). **1**, (*R*)-6-ethyl-2-methyl-2,3-dihydro-4H-pyran-4-one; **2**, (*R*)-2,6-diethyl-2,3-dihydro-4H-pyran-4-one; **4**, (1*R*,3*S*,5*R*)-3-ethyl-1,8-dimethyl-2,9-dioxabicyclo[3.3.1]non-7-en-6-one; **5**, 1,3,8-trimethyl-2,9-dioxabicyclo[3.3.1]non-7-en-6-one; **6**, 1,3-diethyl-8-methyl-2,9-dioxabicyclo[3.3.1]non-7-en-6-one; **7**, (1*R*,3*S*,5*R*)-3-ethyl-1,8-dimethyl-2,9-dioxabicyclo[3.3.1]non-7-ene; **8**, 1,3,8-trimethyl-2,9-dioxabicyclo[3.3.1]non-7-ene; **9**, 1,3-diethyl-8-methyl-2,9-dioxabicyclo[3.3.1]non-7-ene; x, degradation products.

characteristic mass spectra, which were closely related to those of **4** and **7**. In addition to these major bicyclic acetals **4** and **7**, four minor compounds could be identified, the structures of which were found to be represented by the 2,9-dioxabicyclo[3.3.1]nonane system showing the same substitution pattern as

compared to the major components. Two pairs of compounds could be attributed to structures **4** and **7**, respectively: compounds **5** and **8** show a methyl group at positions 1 and 3 while compounds **6** and **9** show ethyl groups at these positions.

Determinations of the absolute configurations of compounds **1**, **2**, **4**, and **7** were carried out by gas chromatography using two different chiral stationary phases. The enantiomers of **1**, **2** and **4** could be separated on a XE-60-L-Val-(*S*)-α-PEA phase, whereas **7** was not resolved (Figure 2). On the other hand, **7** could be well resolved by metal complexation gas chromatography on a Ni(II)-bis(heptafluorobutanoyl-(1*R*,5*S*)-pinan-4-onate) (Schurig and Wistuba, 1984) phase while **1**, **2**, and **4** did not elute, most probably due to complexation to the metal (Figure 3).

Gas chromatographic investigations of the hindleg extracts proved the natural products to be of high enantiomeric purity. Upon comparison with synthetic reference samples, **1** was shown to have 6-*R*-configuration and thus is (*R*)-6-ethyl-2-methyl-2,3-dihydro-4H-pyran-4-one. Since spiking of the natural extract with racemic **2** enhanced the second-eluting enantiomer, which in the case of **1** was attributed to the 6-*R*-enantiomer, we concluded natural **2** to be (*R*)-2,6-diethyl-2,3-dihydro-4H-pyran-4-one. Upon comparison with synthetic reference samples, both natural **4** and **7** proved to show 3-*S*-configuration, and thus are (1*R*,3*S*,5*R*)-3-ethyl-1,8-dimethyl-2,9-dioxabicyclo[3.3.1]non-7-ene and (1*R*,3*S*,5*R*)-3-ethyl-1,8-dimethyl-2,9-dioxabicyclo[3.3.1]non-7-en-6-one. The amounts of **1**, **4**, and **7** per individual were about 40, 5, and 5 μg, respectively. Interestingly, the closely related *H. californicus* was reported to produce an

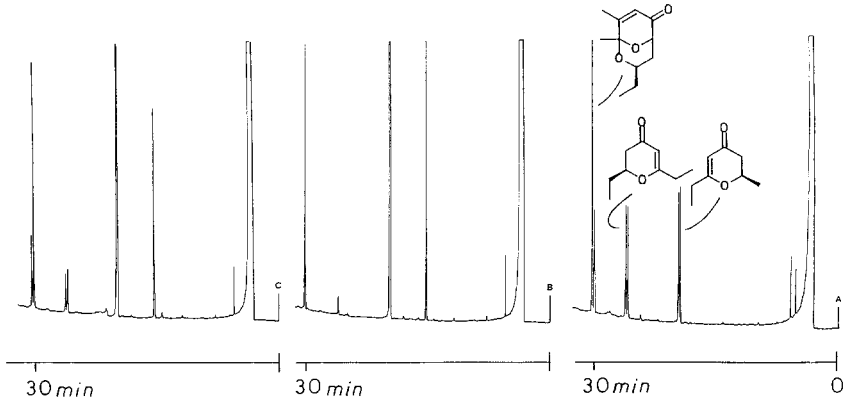


FIG. 2. Gas chromatographic separation of enantiomers of **1**, **2**, and **4** [50-m fused silica capillary coated with XE-60-L-valine-(*S*)-α-phenylethylamide, 0.25 mm ID, programmed from 100°C at 1.5°C/min]. (A) Synthetic mixture of enantiomers; (B) natural sample; (C) mixture of A and B.

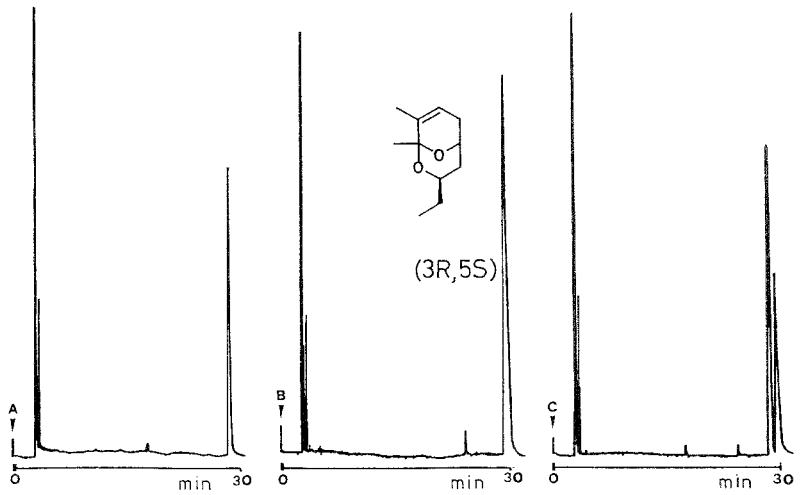


FIG. 3. Gas chromatographic separation of enantiomers of **7** [50-m fused silica capillary coated with Ni(II)-bis(heptafluorobutanoyl-(1*R*,5*S*)-pinan-4-onate) in OV-1, 0.25 mm ID, 80°C isothermal]. (A) Natural sample, (B) synthetic 3*R* enantiomer, (C) mixture of A and B.

isomer of **1**, (*R*)-2-ethyl-6-methyl-2,3-dihydro-4H-pyran-4-one, **3**, as the only volatile compound in their scent legs (Kubo et al., 1985). In contrast to *H. hecta*, no bicyclic acetals were found in the scent glands (Kubo, personal communication).

In addition to *H. hecta*, the secretion of the scent legs of *H. humuli* was investigated. Gas chromatographic analysis of the extracts showed only a single main component, which upon GCMS and comparison of retention times could be identified as (*E*,*E*)-α-farnesene, a widespread naturally occurring sesquiterpene, known from plants and insects. Small amounts of (*E*)-β-farnesene and (*E*,*Z*)- or (*Z*,*E*)-α-farnesene also were found in the extracts (possibly due to decomposition or rearrangement of farnesene in the injection port of the gas chromatograph). No compounds related to **1**, **4**, or **7** could be detected in the extracts.

Field Tests. The behavior of male and female *H. hecta* in response to artifical and natural odor sources was observed in the field. Females were attracted when passing the plume of the male odor during their cruising flight through the habitat. Then they flew upwind to the odor source and landed directly on or close to it. As part of the courtship behavior (Turner, 1976), after landing, a wing fluttering of the females was performed close to the odor source. On 20 evenings when scent sources were tested, more than 50 females showed

the described behavior. These reactions of the females could be observed with all odor source that were tested in the field: freshly cut scent legs, or cotton plugs, which were treated with *n*-pentane extracts of the scent glands or with 1 mg of racemic main component **1**. Control cotton plugs showed no attractancy. Nevertheless, the attractancy of odor sources was restricted to a few meters, even with high concentrations.

Male response to the above odor sources is more variable. Only occasionally did they fly upwind to the source and touch it. Usually they choose a place at some distance (0.5–2 m) downwind to the odor source and started their pendular calling flight ($n > 100$).

Electrophysiology. Twelve males and 15 females were tested. Electroantennograms showed significant responses of male and female antennae to both enantiomers of compounds **1**, **4**, and **7** (see Figure 4A). The absolute amplitudes (mV) of control and odor responses varied with the animals and also slowly changed during the experiments. Nevertheless, relations of response amplitudes remained relatively constant between control and odor stimuli. Due to the short antennae, only small antennogram amplitudes between 2 and 5 mV were recorded even in response to strong odor stimuli, while the control response

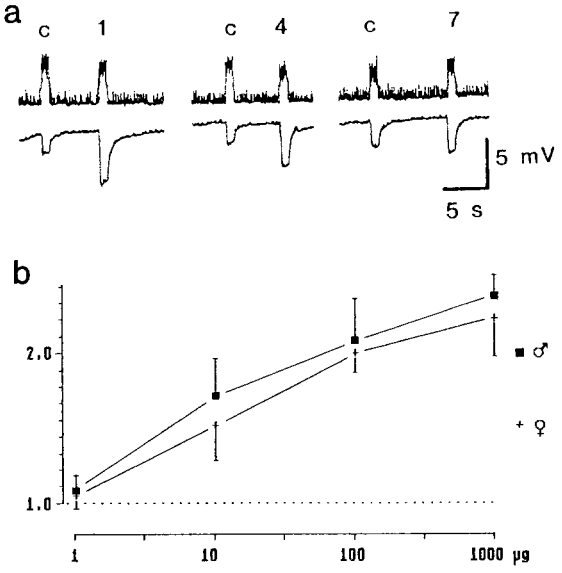


FIG. 4. Electroantennogram recordings. (a) Response of *H. hecta* females to control (c), and 1000 µg of compounds **1**, **4**, and **7** (lower trace). Stimulus duration 1 sec (recorded with an anemometer, upper trace). (b) Dose–response curve from 12 males and 15 females to racemic **1**. Response amplitudes are calculated in ratio to control reactions (= 1). Standard deviations are indicated ($n > 15$).

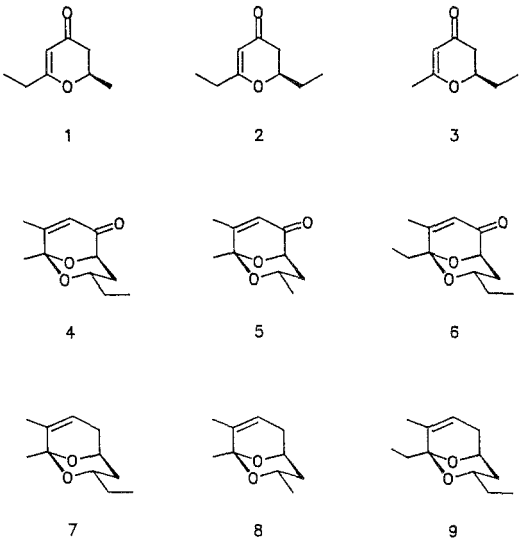


FIG. 5. Absolute configurations of identified compounds.

was only two to three times smaller. A dose–response curve for the male and female antennae to racemic compound **1** is given in Figure 4. Amplitudes are given in relation to the control amplitude set to 1.

The threshold amount for all compounds was between 1 and 10 μg, while saturation was reached at 1000 μg or by whole scent legs as well as their extracts. In general, male and female antennae responded similarly to the stimuli.

Single-cell recordings of female *H. hecta* showed the existence of sensory cells with different sensitivities and reaction patterns to the artificial pheromone components **1**, **4**, and **7**. Some cells showed their best reactivity to component **1** and its optical antipode and were not excited by the components **4** and **7**, while the latter induced significant reactions in other types of sense cells. Details on the complex response pattern will be published elsewhere.

DISCUSSION

The results of chemical analysis and behavioral and electrophysiological investigations revealed that pure enantiomers (*R*)-6-ethyl-2-methyl-2,3-dihydro-4H-pyran-4-one, (1*R*,3*S*,5*R*)-3-ethyl-1,8-dimethyl-2,9-dioxabicyclo[3.3.1]non-7-ene, and (1*R*,3*S*,5*R*)-3-ethyl-1,8-dimethyl-2,9-dioxabicyclo[3.3.1]non-7-en-6-one make up the sex pheromone of male *H. hecta*. These compounds are not reported from other Lepidoptera. Both acetals belong to a new class of biologically active insect volatiles.

Electroantennograms and single-cell recordings demonstrate that all three components are biologically active. Pure compound **1**, as well as the whole bouquet, elicits the same sequences of the female mating behavior, with initial attraction and wing fluttering. Nevertheless, the additional components might play a role during the final part of the mating behavior, i.e., between landing of the male and the completion of copulation, or during the ("agonistic"?) behavioral interactions between males (Turner, 1988).

From the biogenetic point of view, dihydropyrone **1** of *H. hecta* is closely related to the dihydropyrone **3** of *H. californicus*. Both acetogenins should have the same biogenetic precursor, possibly a triply oxidized C_8 unit, which is differently cyclicized in the two species, yielding the respective dihydropyrones.

While **4** and **7** may share a biosynthetic precursor, there is no obvious biogenetic relation to **1**. In contrast to **1**, both compounds possess a branched carbon skeleton, which points to a mixed acetate–propionate biosynthesis. This trialkyl-2,9-dioxabicyclo[3.3.1]nonane system is found in nature only seldom, so that a specific informative function of these molecules is more than likely.

In contrast to the rather stereotypical attractance of male moths by their female pheromones, male lepidopteran pheromones show a variety of functions (Boppré, 1984). Attraction of females—as now found in *Hepialus*—is usual (see Wunderer et al., 1985). Like other male pheromone systems (Schneider, 1984), the gland of male *H. hecta* contains an amount of pheromone much higher than found in female calling systems. Nevertheless, the range of attractancy in this calling system is restricted to several meters only.

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EFFECTS OF UREA ANALOGS ON EGG HATCHING AND MOVEMENT OF UNHATCHED LARVAE OF MONOGENEAN PARASITE *Acanthocotyle lobianchi* FROM SKIN OF *Raja montagui*

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Abstract—Rapid hatching in the monogenean parasite *Acanthocotyle lobianchi* from the skin of *Raja montagui* is stimulated by urea. Structurally similar to the urea molecule, the following analogs of urea provide amino groups, carboxyl groups, or combinations of these, but fail to stimulate hatching at concentrations of 1 mM in seawater: methylurea (MU); 1,3-dimethylurea (DU); 1,1,3,3-tetramethylurea (TMU); thiourea (TU); 1,1,3,3-tetramethyl-2-thiourea (TMTU); and 1-phenyl-2-thiourea (PTU). All of these analogs except PTU elicit movements of unhatched larvae, and posttreatment of the eggs with urea showed that the ability to hatch is not impaired by initial treatment with any of the urea analogs. Thus the larval chemoreceptor that initiates hatching appears to be highly specific for the urea molecule.

Key Words—*Acanthocotyle lobianchi*, monogenean, *Raja montagui*, elasmobranch, parasite eggs, oncomiracidia, hatching factor, urea, urea analogs, chemoreceptors.

INTRODUCTION

Fully embryonated eggs of the monogenean skin parasite *Acanthocotyle lobianchi* rarely hatch when incubated in seawater, and larvae within the eggs may

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remain alive for up to 83 days at 13°C (Macdonald, 1974). However, mucus from the skin of the host (*Raja* spp.) induces rapid hatching (within 2–4 sec). Kearn and Macdonald (1976) found that seawater containing analytical grade urea, at concentrations similar to those in ray skin mucus, also stimulates hatching in *A. lobianchi*. Their finding that the stimulatory effect of ray skin mucus is destroyed by incubation with urease and restored by the addition of urea crystals, indicates that urea is an important hatching factor derived from the host. Factors such as mechanical disturbance and reductions in light intensity, which are known to stimulate hatching in other monogeneans (see Whittington and Kearn, 1988, 1989), fail to stimulate hatching in *A. lobianchi* (see Macdonald, 1974), and the apparent dependence of this parasite on a single hatching stimulant provides a useful experimental system for the investigation of chemoperception. As a starting point, it was decided to test the effectiveness of commercially available analogs of urea as hatching stimulants for *A. lobianchi*, in an attempt to throw light on the molecular interaction between the stimulant and the sensory receptors of the oncomiracidium.

METHODS AND MATERIALS

Collection and Maintenance of Eggs. Living adult specimens of *Acanthocotyle lobianchi* were collected from the skin of freshly landed *Raja montagui* caught by the research vessels of the Marine Biological Association of the United Kingdom at Plymouth. On some occasions, living, egg-laying parasites from *R. montagui* were sent to Norwich in vacuum flasks of cooled seawater. Groups of 5–15 adult parasites were maintained in covered glass crystallizing vessels (5 cm diameter) containing about 30 ml of Plymouth seawater (PSW) filtered through two sheets of Whatman No. 1 filter paper to remove marine organisms that might be a source of urea. The PSW was changed each day. Eggs were collected and incubated in crystallizing vessels following a procedure similar to that used by Macdonald (1974) and Kearn and Macdonald (1976) in previous studies on *A. lobianchi* eggs. Briefly, eggs were kept at 13–14°C and exposed to 12-hr periods of alternating dim blue light and darkness (LD 12 : 12; light on, 0930 hr; light off, 2130 hr) selected to simulate conditions on the seabed at depths where rays are found (Whittington and Kearn, 1986). The PSW was changed daily during incubation and, at the same time, the eggs were examined for signs of embryonation and hatching.

Hatching Tests with Urea analogs. The analogs of urea chosen for testing were: methylurea (MU); 1,3-dimethylurea (DU); 1,1,3,3-tetramethylurea (TMU); thiourea (TU); 1,1,3,3-tetramethyl-2-thiourea (TMTU); and 1-phenyl-2-thiourea (PTU). The chemical structures of these substances and that of urea are shown in Figure 1. MU, DU, TMTU, and PTU were obtained from Sigma

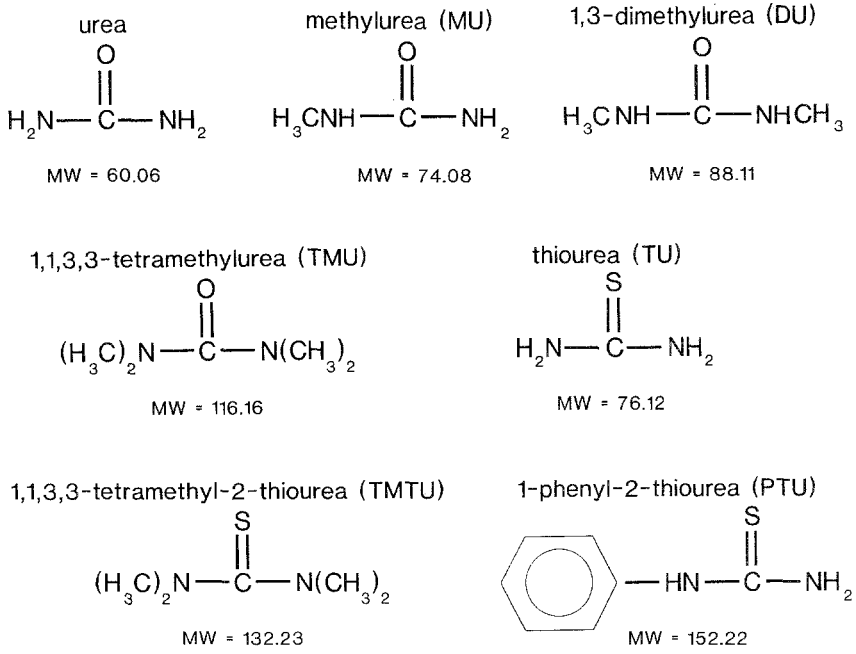


FIG. 1. The chemical structures of urea and of the urea analogs used in hatching studies on eggs of *Acanthocotyle lobianchi*. The molecular weight (MW) of each chemical is shown beneath each structure.

Chemical Co. and Analar urea (analytical grade crystals, minimum assay—99.5% urea; see below), TMU, and TU from BDH Chemicals. A 1 mM solution was chosen for each urea analog tested because a 1 mM solution of urea in seawater readily hatches *A. lobianchi* eggs and estimates of the urea concentration in mucus from the ventral skin of *Raja clavata* range from 0.8 to 1.5 mM (Kearn and Macdonald, 1976). Before use, each test solution made up in PSW was allowed to reach the same temperature as the PSW containing the eggs.

Three experiments were performed with each analog using eggs containing fully developed larvae of three different ages, namely 27, 46, and 75 days (eggs reach full development after about 15 days at 13°C, according to Macdonald, 1974). Each experiment was carried out by transferring 5–20 eggs of the appropriate age to about 10 ml of the test solution in a Syracuse watch glass. The transfer was carried out using a chemically clean pipet drawn fine enough to minimize the amount of seawater transferred with the eggs. This procedure was performed during the 12-hr period of illumination on the stage of a stereomi-

croscope with dim illumination from below. Eggs were observed continuously for 15 min for signs of either larval movement or hatching and were then removed from the test solution and transferred for posttreatment to a second watch glass containing approximately 10 ml of a 1 mM solution of Analar urea in PSW. Posttreated eggs then were observed for 15 min. With each experiment, controls were treated first for 15 min with PSW and then posttreated for 15 min with a 1 mM solution of Analar urea in PSW.

RESULTS

No hatching occurred when eggs containing fully developed and living larvae were incubated in clean PSW for up to 75 days and no hatching occurred as a result of treatment of fully developed eggs aged between 27 and 75 days with any of the urea analogs (Table 1, experiments 1–18). Larval movements were observed in some eggs aged 27, 46, and 75 days when treated with MU, DU, TMU, TU, and TMTU (Table 1, experiments 1–3, 4–6, 7–9, 10–12, and 13–15 respectively). No larval movements were seen in any of the eggs treated with PTU (Table 1, experiments 16–18) or in the eggs in the control batches treated initially with PSW. When test and control batches were posttreated with a 1 mM solution of Analar urea in PSW, between 42.9 and 100% of the eggs tested in each batch hatched within 5 sec. No further hatching occurred during the rest of the posttreatment period, but in the unhatched eggs, irrespective of age and previous treatment, movements of larvae were observed. Posttreatment of 75-day-old eggs with urea, after initial treatment with MU, DU, TMU, TU (Table 1, experiments 3, 6, 9, 12) and PSW (control), elicited 100% hatching, but this was not the case when 75-day-old eggs were posttreated with urea after initial treatment with TMTU and PTU. With TMTU (experiment 15), 71.4% of the eggs hatched and with PTU (experiment 18) 42.9% hatched.

DISCUSSION

As pointed out by Kearn and Macdonald (1976), it seems likely that the small urea molecule diffuses readily through the eggshell (or through the opercular seal) of *Acanthocotyle lobianchi*. Presumably the urea molecule binds to superficial receptors of the oncomiracidium and elicits a neuromuscular response, which, as observed by Macdonald (1974), leads to sudden extension of the body of the larva and dislodgement of the operculum. It has been shown in the present study that several analogs of urea, namely, MU [molecular weight (MW) = 74.08], DU (MW = 88.11), TMU (MW = 116.16), TU (MW = 76.12), TMTU (MW = 132.23), and PTU (MW = 152.22), all at concentrations of 1 mM in PSW, do not stimulate hatching in fully developed eggs rang-

TABLE 1. EFFECT OF UREA ANALOGS ON EGG HATCHING AND ACTIVITY OF UNHATCHED LARVAE OF *Acanthocotyle lobianchi*[a]

Expt	Initial treatment (1 mM solutions of urea analogs)	Age of eggs (days)	No. of eggs tested	No hatched in initial treatment	Movements within egg	Hatch in initial treatment %	Hatch in posttreatment No.	Hatch in posttreatment %
1	MU	27	12	0	+	0	10	83.3
2	MU	46	10	0	+	0	7	70
3	MU	75	8	0	+	0	8	100
4	DU	27	14	0	+	0	10	71.4
5	DU	46	10	0	+	0	10	100
6	DU	75	8	0	+	0	8	100
7	TMU	27	6	0	+	0	3	50
8	TMU	46	5	0	+	0	3	60
9	TMU	75	6	0	+	0	6	100
10	TU	27	20	0	+	0	14	70
11	TU	46	7	0	+	0	6	85.7
12	TU	75	6	0	+	0	6	100
13	TMTU	27	5	0	+	0	5	100
14	TMTU	46	6	0	+	0	5	83.3
15	TMTU	75	7	0	+	0	5	71.4
16	PTU	27	17	0	−	0	14	82.4
17	PTU	46	9	0	−	0	8	88.9
18	PTU	75	7	0	−	0	3	42.9
	Control (PSW)	27	11	0	−	0	9	81.8
	Control (PSW)	46	8	0	−	0	8	100
	Control (PSW)	75	7	0	−	0	7	100

[a] Only one set of controls for eggs of each age group is shown. +, movements of larvae observed within some eggs; −, no movements of larvae observed within any eggs.

ing in age from 27 to 75 days. That this is not the consequence of an irreversible effect on the ability of the larvae to hatch was demonstrated by the observation that between 42.9 and 100% of the posttreated eggs hatched in the presence of a 1 mM solution of Analar urea in PSW.

The molecular weights of all the analogs of urea used in these experiments were greater than that of urea (Figure 1), and it is possible that some or all of these substances diffuse too slowly into the egg to stimulate the larva or completely fail to penetrate. However, some degree of penetration by most of these chemicals seems likely because, with the exception of PTU, which has the largest molecular weight of the chemicals tested, all the analogs stimulated movements of larvae within the eggs. If it is assumed that the analogs are able to enter the intact egg, then some inferences can be drawn about the nature of the chemoreceptor responsible for the initiation of the hatching behavior. The lack of a hatching response to TU and PTU suggests that the amino group in the urea molecule is not alone responsible for activating the chemoreceptor and, similarly, the failure to respond to DU and TMU suggests it is unlikely that the active part of the urea molecule is the carboxyl group. Molecules such as MU with both an amino group and a carboxyl group also appear to be ineffective as hatching stimulants, and Kearn and Macdonald (1976) showed that substances likely to be excreted by elasmobranchs, such as trimethylamine oxide, ammonium chloride, and some amino-acids, fail to stimulate hatching. Thus, the oncomiracidial chemoreceptor responsible for initiating the hatching process appears to have a high specificity for the urea molecule, failing to respond to molecules similar to urea with amino groups only, with carboxyl groups only, or with both of these groups.

It is interesting that all of the analogs of urea except PTU appear to stimulate movement of the larva but fail to trigger the extension of the body of the larva that dislodges the operculum. It is possible that urea-mediated hatching is a two-stage process involving, first, activation of the larva and, second, extension of the body. Perhaps the urea analogs (with the exception of PTU) initiate the first stage but fail to stimulate extension of the body of the larva.

Whittington (1987) determined that eggs of the microbothriid *Leptocotyle minor* and of the hexabothriid *Hexabothrium appendiculatum* from the skin and gills, respectively, of the same elasmobranch host, *Scyliorhinus canicula*, also hatch rapidly (within 10–50 sec) after application of urea. However, eggs of *L. minor* also hatch after treatment with a 100 mM solution of TU in PSW but not with a 5 mM solution of TU in PSW. He suggested that the observed hatching response of *L. minor* eggs to relatively high concentrations of TU might indicate a disposition for thiourea to bind to receptor sites which usually bind urea, or that the eggs might have responded to an impurity, possibly urea, in the commercial sample of thiourea. In the present study, eggs of *A. lobianchi* were not tested with concentrations of urea analogs other than 1 mM, and it remains to

be determined whether higher concentrations of these chemicals stimulate hatching.

Kearn and Macdonald (1976) found that the susceptibility of eggs of *A. lobianchi* to stimulation with urea appears to increase with the age of the egg. A similar trend was detected in the present study. However, despite the enhanced capacity of the 75-day-old eggs to respond to urea, none reacted to any of the analogs tested. Curiously, not all 75-day-old eggs treated initially with TMTU and PTU hatched on posttreatment with urea. This may indicate some toxicity of TMTU and PTU for older eggs, but toxicity does not seem to be a feature of TU treatment, at least at a concentration of 1 mM. This contrasts with the finding of Simpson and Ogden (1932) that TU is toxic to the elasmobranch heart.

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BENEFICIAL ARTHROPOD BEHAVIOR MEDIATED BY AIRBORNE SEMIOCHEMICALS. IX. DIFFERENTIAL RESPONSE OF *Trichogramma pretiosum*, AN EGG PARASITOID OF *Heliothis zea*, TO VARIOUS OLFACTORY CUES

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Abstract—The behavior of *Trichogramma pretiosum* Nixon wasps when exposed to different olfactory cues was studied in a wind tunnel. Compared to clean air, the sex pheromone of its host *Heliothis zea* (Boddie) increased wasp residence times, walking times, and path lengths on a platform and decreased walking velocity. If wasps were released on top of a glass rod above a platform, the odor caused the wasps to land shortly after takeoff. In addition, a clear dose effect with regard to total residence and walking times was found. These responses were not elicited by three dosages of the sex pheromone of *Spodoptera frugiperda* (J.E. Smith) or by a blend of saturated acetates. These results correspond with the observation that *H. zea* is a common field host of *T. pretiosum*, whereas eggs of *S. frugiperda* are rarely attacked by this parasitoid.

Key Words—*Trichogramma pretiosum*, Hymenoptera, Trichogrammatidae, *Heliothis zea*, *Spodoptera frugiperda*, Lepidoptera, Noctuidae, sex pheromone, kairomone, wind tunnel, orientation behavior.

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INTRODUCTION

Olfactory receptor systems of insects are shaped continuously by sensory adaptation and natural selection to perceive chemical cues (i.e., semiochemicals) in an ever-changing environment. Of particular significance for foraging parasitic wasps are host-derived kairomones, the function of which has now been demonstrated for many species of parasitoids in a variety of taxonomic groups (Vinson, 1984, 1985; Noldus, 1989c). Here we focus on cases where the kairomone used by a parasitoid is part of the intraspecific communication system of the host, so that the parasitoid can be labeled as a "chemical eavesdropper" or "chemical spy." Examples are known from several parasitoids of bark beetles that respond to their host's aggregation pheromone (Wood, 1982). A similar phenomenon has been demonstrated recently for egg parasitoids of the genus *Trichogramma*. These insects have received—and continue to receive—considerable research attention, due mainly to their potential as biological control agents against lepidopteran crop pests (Voegelé et al., 1988). *Trichogramma pretiosum* Riley, for example, is a candidate for inundative releases against *Heliothis* spp. (King et al., 1986; Ridgway et al., 1988; King and Coleman, 1989).

Initial field and greenhouse observations (Lewis et al., 1982) and subsequent olfactometer experiments (Noldus, 1988) indicated that female *T. pretiosum* utilize the sex pheromone of *H. zea* as a kairomone in their search for host eggs. We recently analyzed the orientation behavior of two *Trichogramma* spp. (*T. evanescens* Westwood and *T. pretiosum*) in response to the sex pheromone of their noctuid hosts [*Mamestra brassicae* L. and *Heliothis zea* (Boddie)] in a wind tunnel (Noldus et al., 1990a). Orientation by wasps to host sex pheromone was achieved by a combination of odor-modulated phototaxis and odor-induced inverse orthokinesis. Compared to clean air, kairomone-loaded air decreased walking velocity and increased residence times, walking times, and path lengths on a platform. During locomotion on a horizontal platform, upwind anemotaxis was evident but remained unaffected by odor. On a platform inclined 45°, the lower edge facing upwind, anemotaxis appeared to be offset by positive phototaxis. If wasps were released on top of a glass rod above a platform, the kairomone caused wasps to land shortly after takeoff.

The sex pheromone of *H. zea* is a mixture of four straight-chain even-carbon-numbered aldehydes. The synthetic blend identified as the most effective sex attractant in the field consisted of (*Z*)-11-hexadecenal (Z11–16 : Al), (*Z*)-9-hexadecenal (Z9–16 : Al), (*Z*)-7-hexadecenal (Z7–16 : Al) and hexadecanal (S–16 : Al), in a 87 : 3 : 2 : 8 ratio (Klun et al., 1980). This blend also was used in the previous experiments with *Trichogramma* (Lewis et al., 1982; Noldus et al., 1989a). Given the fact that *T. pretiosum* is found on several species of Lepidoptera in the field (Pinto et al., 1986), one may wonder if *T. pretiosum*

will respond to any randomly chosen blend of straight-chain aliphatic compounds.

Further, the responses of *T. pretiosum* to the sex pheromone of *H. zea* recorded so far were obtained in laboratory setups, either a four-arm airflow olfactometer (Noldus, 1988) or a wind tunnel (Noldus et al., 1990a), in which host pheromone was tested against clean air. In the absence of other stimuli for comparison, responses to stimuli recorded in such assays might represent a response to something vs. nothing, rather than a host-directed response (Jones, 1986).

To investigate these two hypotheses, comparative wind tunnel experiments were carried out in which *T. pretiosum* was exposed to the sex pheromone blend of *H. zea* and to (1) a biologically active sex pheromone blend of another moth species, but nonhost of *T. pretiosum*, and (2) an artificial blend of acetates.

As the first test substance, we used the sex pheromone of the fall armyworm moth, *Spodoptera frugiperda* (J.E. Smith). This was chosen because both *S. frugiperda* and *H. zea* are noctuids, with similar geographic distributions and substantial overlap in host-plant ranges (Harding, 1976; Sparks, 1979; Hill, 1987). However, *S. frugiperda* is not a host of *T. pretiosum*. Calling female *S. frugiperda* release a five-component blend, consisting of (*Z*)-7-dodecen-1-yl acetate (Z7–12 : Ac) (3.2%), 11-dodecen-1-yl acetate (11–12 : Ac) (22%), dodecan-1-yl acetate (S–12 : Ac) (1.9%), (*Z*)-9-tetradecen-1-yl acetate (Z9–14 : Ac) (90.1%) and (*Z*)-11-hexadecen-1-yl acetate (Z11–16 : Ac) (2.6%) (Tumlinson et al., 1986).

The second test material was a mixture that was chemically related to lepidopteran sex pheromones but that *T. pretiosum* would not encounter in the field. We used a blend of three saturated acetates—dodecanyl acetate (S–12 : Ac), tetradecanyl acetate (S–14 : Ac) and pentadecanyl acetate (S–15 : Ac)—that has no known function as a semiochemical.

METHODS AND MATERIALS

Parasitoid Rearing. Trichogramma pretiosum wasps were reared on eggs of *H. zea* as described by Noldus (1988). *Heliothis* eggs were obtained from a laboratory culture; they had been treated with a solution of sodium hypochlorite for removal of adhesives and other chemicals and for sterilization (Burton, 1969). Only 2-day-old female wasps were used. To reduce interindividual behavioral variation they were allowed to oviposit in a *H. zea* egg ca. 1 hr prior to the experiment and were then isolated for ca. 30 min (Noldus, 1988; 1989b).

Odor Sources. The following odor sources were used in the experiments:

1. The synthetic sex pheromone of *H. zea*. The blend consisted of a 87 : 3 : 2 : 8 mixture of Z11–16 : Al, Z9–16 : Al, Z7–16 : Al and S–16 : Al. It was

loaded on rubber septa at dosages of 0.1, 1, and 10 mg. These high dosages were necessary to obtain release rates in a range covering that of a calling female, at an airflow of 500 ml/min over the septum. A septum loaded with 1 mg released ca. 55 ng/hr, as determined by collection of volatiles and analysis by gas chromatography using the method of Heath et al. (1986). This is close to the rate at which the major component Z11–16 : Al is released during peak emission (Pope et al., 1984).

2. The synthetic sex pheromone of *S. frugiperda*. The blend tested was loaded on rubber septa in such a ratio as to release the five compounds in the approximate ratio as they are released by calling females. It consisted of Z7–12 : Ac (0.6%), 11–12 : Ac (0.7%), S–12 : Ac (0.4%), Z9–14 : Ac (87.0%) and Z11–16 : Ac (11.3%), loaded on septa at dosages of 0.1, 1, and 3 mg.

3. A blend of three saturated acetates (S–12 : Ac, S–14 : Ac and S–15 : Ac) in a 33 : 33 : 34 mixture. This blend was tested at a dosage of 1 mg/septum.

The desired amount of a blend in 200 μl hexane was pipetted into the large well of a rubber septum and the solution was allowed to soak into the septum. Then the septum was aired under a laboratory hood for 48 hr to remove the solvent. Before a test, septa were aired for 1 hr, and between tests they were stored at room temperature.

Wind Tunnel Experiment. We used a horizontal wind tunnel similar to the one described elsewhere (Noldus et al., 1990a), with minor modifications. Air was blown over the septum at a rate of 500 ml/min, of which 70% was discarded via a three-way stopcock directly downwind of the container holding the septum. Odor was injected into the main stream by blowing the odor-laden air through a glass nozzle (made from a Pasteur pipet, cf. Zanen et al., 1989) at a rate of 150 ml/min. With the prevailing ventilator speed (which produced a main stream of ca. 5 cm/sec), this resulted in a turbulent jet plume with a diameter of ca. 10 cm and an airspeed of ca. 15 cm/sec in the center at 45-cm downwind from the opening of the nozzle (measured with a hot-wire anemometer). At this point, a platform was so located as to be completely enveloped by the plume. The design of experiments was based on a combination of overhead light and airborne chemicals as competing stimuli for *Trichogramma* wasps (see Noldus et al., 1989a). Overhead illumination was provided by four fluorescent tubes, which gave a light intensity of ca. 5.9 W/m^2 at the center of the platform.

At the start of a test, a septum loaded with test chemicals was placed in a brass container, which was connected to the nozzle, after which the airflow was started. Observations were begun after the pump had run for at least 20 min, to allow the odor concentration to stabilize. Treatments were alternated in a systematic fashion. Between treatments, the wind tunnel was rinsed with ethanol and clean air was drawn through at 50 cm/sec for at least 2 hr. After a day of testing, the wind tunnel was rinsed with ethanol and ventilated with clean air

until the following day. The behavior of the insects was recorded on a TRS-80 Model 100 microcomputer, programmed as an event recorder (cf. Noldus, 1990). All experiments were performed at 29 ± 1°C and 60 ± 10% relative humidity.

Walking Experiments. Two types of experiments were conducted. In both, platforms were used as experimental arena in order to mimic leaves. In the first, referred to as walking experiments, the experimental arena consisted of a platform, inclined at 45°, with the lower edge facing the wind. The center of the platform was 15 cm above the floor of the wind tunnel. It was made of white cardboard covered with translucent plastic and had a surface area of 5 × 5 cm. A thin streak of odorless petroleum jelly along the edge underneath the platform prevented the wasps from walking to the lower surface. The platform was cleaned with ethanol after every observation. Wasps were released individually in the center of the platform and were observed until they flew away or until 10 min had elapsed. The distance between the observer and the wasps hampered detailed inspection of their behavior, which was therefore classified as either walking or not walking. In order to record the position of the insect, a grid of 25 square sectors (1 × 1 cm) was drawn on the platform, and behavioral events were recorded separately for each sector. The following parameters were measured: total time on the platform, time spent walking, proportion of time spent on the upwind half of the platform, path length [estimated by counting the total number of platform sectors traversed (cf. Reddingius et al., 1983); as this parameter was only used for comparative purposes, no conversion factor was applied to estimate the distance actually covered], walking velocity (estimated total path length divided by absolute walking time), and number of different platform sectors traversed [when compared to the total number of sectors (25), this can be used as a crude index of the proportion of the platform traversed].

Flight Experiments. The second type of experiment, referred to as flight experiments, were designed to test the effect of various odors on *Trichogramma*'s behavior at the onset of flight. Wasps were released individually on top of a glass rod (4 mm diam.), extending 2 cm above the center of a green horizontal platform (6 × 6 cm), with a 2-mm-wide ring of odorless petroleum jelly around the rod (1 mm from the top) preventing wasps from walking down the rod. Flights could be categorized into upward flights towards the overhead light (which resulted in a landing on the ceiling or—very rarely—on the side wall of the tunnel), and flights resulting in a landing on the platform, providing an assay to test the effect of odor on: latency to takeoff, percentage of individuals landing on the platform, and frequency of landings on the upwind and downwind half of the platform. Parasitoids that did not take off from the rod within 5 min were discarded (ca. 1% of the trials). After every observation the rod was cleaned with ethanol to prevent accumulation of odor molecules or traces of the wasps.

Statistical Analysis. Frequencies and durations of behaviors were com-

puted with The Observer, a software package for behavioral research (Noldus, 1989a, 1990). All data were tested for normality, using the Shapiro-Wilk test (if $N \leq 50$) or the Kolmogorov test (SAS, 1985). The distribution of most parameters deviated significantly from a normal distribution. Those parameters were analyzed with nonparametric statistical tests. Otherwise parametric tests were used. In the dose–response experiments, the dosages were compared to test for a dose effect; in a number of cases values of a single dosage were tested against those obtained with clean air. Further details are given as footnotes in the tables.

RESULTS

Response to Various Olfactory Cues. In the first walking experiment, parasitoids were exposed to three different odors and clean air. All odors were loaded on septa at a dosage of 1 mg. As shown in Table 1, only the sex pher-

TABLE 1. BEHAVIOR OF INDIVIDUAL *Trichogramma pretiosum* WASPS ON PLATFORM IN WIND TUNNEL WHEN EXPOSED TO VARIOUS STIMULI[a]

Parameter	Stimulus: Clean air	Sex pheromone *Heliothis zea*	Sex pheromone *Spodoptera frugiperda*	Acetate blend	P^b
Number of observations	50	50	50	50	
Total time on platform (sec)	99.9 ± 15.6[c]a	186.3 ± 22.8b	101.0 ± 15.3a	73.7 ± 16.9a	**
Time spent walking (%)	71.5 ± 3.1a	86.3 ± 2.6b	72.5 ± 2.9a	78.2 ± 4.7a	**
Estimated path length (number of sectors traversed)	17.9 ± 2.8a	30.3 ± 3.4b	15.6 ± 1.6a	12.8 ± 1.4a	**
Walking velocity (number of sectors traversed/sec)	0.33 ± 0.02a	0.24 ± 0.02b	0.35 ± 0.02a	0.41 ± 0.02a	**
Number of different sectors traversed	11.2 ± 0.9a	15.7 ± 1.0b	10.9 ± 0.8a	9.0 ± 0.7a	**
Time spent in upwind half (%)	53.3 ± 4.4a	44.3 ± 3.5ab	35.4 ± 3.8b	45.9 ± 4.4ab	*
Wasps spending more than 50% time in upwind half (%)	58.0 (n.s.)[d]	38.0 (n.s.)	30.0 (**)	32.0 (*)	

[a] The platform measured 5 × 5 cm, and was divided into 25 1 × 1-cm sectors.
[b] Kruskal-Wallis test (SAS, 1985), followed by distribution-free multiple comparison (Hollander and Wolfe, 1973). Different letters in a row indicate significant differences. n.s.: $P > 0.05$; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.
[c] Mean ± SE.
[d] In parentheses: 2-tailed significance level of difference from 50% (sign test) (Siegel, 1956).

omone of *H. zea* elicited responses. These were typical of those found previously (Noldus et al., 1990a): increased total residence and walking times, path length, and proportion of platform traversed and reduced walking velocity. The only significant effect of the sex pheromone of *S. frugiperda* was a tendency for wasps to spend proportionally more time in the downwind half of the platform, compared to clean air.

Similar results were obtained in the first flight experiment (Table 2): unlike the sex pheromone of *H. zea*, the other odors did not alter the percentage of wasps landing on the platform. Latency and the frequency of landings on upwind and downwind halves of the platform remained unaffected by the odor offered. In no treatment did the percentage of individuals landing on a given side differ significantly from 50%.

Dose-Response Relationships. In the previous experiments, *T. pretiosum* responded to the sex pheromone of *H. zea* but not to that of *S. frugiperda*. However, both stimuli were compared at a single dosage, and a response to the fall armyworm sex pheromone might be obscured by a different dose-response relationship. The lack of response to the sex pheromone of *S. frugiperda* might thus have been due to the dosage tested. In addition, the sex pheromone of *H. zea* had been tested at ca. 30% of the concentration released by individual calling females. However, *T. pretiosum* is likely to be exposed to the kairomone in the field at a different concentration. Therefore, we examined the behavior

TABLE 2. FLIGHT BEHAVIOR OF *Trichogramma pretiosum* WASPS IN WIND TUNNEL WHEN EXPOSED TO VARIOUS STIMULI

Parameter	Stimulus				
	Clean air	Sex pheromone *Heliothis zea*	Sex pheromone *Spodoptera frugiperda*	Acetate blend	P^a
Number of observations	100	100	100	100	
Latency to takeoff (sec)	42.9 ± 5.8[b]	29.1 ± 4.1	35.3 ± 5.2	39.0 ± 5.4	n.s.
Individuals landing on platform (%)	17.0 ± 11.2[c]a	46.0 ± 18.5b	13.0 ± 5.1a	13.0 ± 5.1a	**
Individuals landing on upwind half of platform (%)	38.4 ± 7.4[b] (17)[d] [n.s.][e]	50.9 ± 5.8 (46) [n.s.]	48.3 ± 15.7 (13) [n.s.]	33.3 ± 16.3 (13) [n.s.]	n.s.

[a] n.s.: $P > 0.05$; *: $P < 0.05$; **: $P < 0.01$.
[b] Mean ± SE. Differences tested with Kruskal-Wallis test (STSC, 1986).
[c] Mean ± SD of five blocks of 20 observations. Different letters indicate significant differences (95% LSD intervals) (STSC, 1986).
[d] In parentheses: number of landings of which position was recorded.
[e] In brackets: two-tailed significance level of difference from 50% (sign test).

of *T. pretiosum* when exposed to clean air vs. various concentrations of the pheromones of *H. zea* and *S. frugiperda*, respectively. With increasing concentrations of the sex pheromone of *H. zea* we observed a significant increase in total residence and walking times (Table 3). Other parameters (estimated path length, number of different sectors traversed) also showed an upward trend, but means were not significantly different. Furthermore, all parameters differed significantly from values obtained in clean air, as followed from paired tests with the lowest dosage. In contrast, dose–response relationships with *S. frugiperda* were insignificant for all parameters, except for walking velocity, which was higher with the highest dosage tested (Table 4). This value was also higher than the value obtained in clean air.

Similarly, in flight experiments we also found a significant dose response with the sex pheromone of *H. zea* (Table 5) and not with that of *S. frugiperda* (Table 6). With the pheromone of *H. zea*, increasing dosages yielded increasing percentages of wasps landing on the platform, and the value obtained with the lowest dosage (0.1 mg) was significantly different from that found in clean air. In contrast, the concentration of pheromone of *S. frugiperda* did not affect the percentage of wasps landing. With both odors, time until takeoff and the distribution of landings over upwind and downwind halves of the platform were

TABLE 3. BEHAVIOR OF INDIVIDUAL *Trichogramma pretiosum* WASPS ON PLATFORM IN WIND TUNNEL WHEN EXPOSED TO DIFFERENT DOSAGES OF SYNTHETIC SEX PHEROMONE OF *Heliothis zea*

Parameter	Clean air	Sex pheromone dosage (mg)			P^a
		0.1	1	10	
Number of observations	100	100	100	100	
Total time on platform (sec)	97.2 ± 13.9[b]	117.2 ± 13.4a (***)[c]	132.0 ± 16.6a	174.3 ± 17.5b	*
Time spent walking (%)	68.7 ± 2.3	87.4 ± 1.5	86.1 ± 1.8	85.8 ± 1.8	n.s.
Path length (number of sectors traversed)	15.0 ± 1.7	21.4 ± 2.0	22.9 ± 2.8	31.8 ± 3.3	n.s.
Walking velocity (number of sectors traversed/sec)	0.40 ± 0.03	0.26 ± 0.01	0.26 ± 0.01	0.25 ± 0.01	n.s.
Number of different sectors traversed	9.8 ± 0.6	12.6 ± 0.6	12.6 ± 0.6	14.2 ± 0.6	n.s.

[a] Kruskal-Wallis test for differences between dosages (i.e., clean air not included), followed by distribution-free multiple comparison. Different letters in a row indicate significant differences, n.s.: $P > 0.05$; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

[b] Mean ± SE.

[c] In parentheses: two-tailed significance level of difference with clean air (Mann-Whitney U test).

TABLE 4. BEHAVIOR OF INDIVIDUAL *Trichogramma pretiosum* WASPS ON PLATFORM IN WIND TUNNEL WHEN EXPOSED TO DIFFERENT DOSAGES OF SYNTHETIC SEX PHEROMONE OF *Spodoptera frugiperda*

Parameter	Clean air	Sex pheromone dosage (mg) 0.1	1	3	P^a
Number of observations	50	50	50	50	
Total time on platform (sec)	99.9 ± 15.6[b]	85.6 ± 14.1	101.0 ± 15.3	108.7 ± 17.9	n.s.
Time spent walking (%)	71.5 ± 3.1	61.6 ± 3.3	72.5 ± 2.9	64.4 ± 3.5	n.s.
Path length (number of sectors traversed)	17.9 ± 2.8	14.5 ± 2.3	15.6 ± 1.6	21.2 ± 3.1	n.s.
Walking velocity (number of sectors traversed/sec)	0.33 ± 0.02	0.40 ± 0.04 (n.s.)[c]ab	0.35 ± 0.02 (n.s.)a	0.40 ± 0.02 (***)b	*
Number of different sectors traversed	11.2 ± 0.9	9.4 ± 0.8	10.9 ± 0.8	12.1 ± 0.9	n.s.

[a] Kruskal-Wallis test for differences between dosages (i.e., clean air not included), followed by distribution-free multiple comparison. Different letters in a row indicate significant differences. n.s.: $P > 0.05$; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.
[b] Mean ± SE.
[c] In parentheses: two-tailed significance level of difference with clean air (Mann-Whitney U test).

TABLE 5. FLIGHT BEHAVIOR OF *Trichogramma pretiosum* WASPS IN WIND TUNNEL WHEN EXPOSED TO DIFFERENT DOSAGES OF SYNTHETIC SEX PHEROMONE OF *Heliothis zea*

Parameter	Clean air	Sex pheromone dosage (mg) 0.1	1	10	P^a
Number of observations	150	150	150	100	
Latency to takeoff (sec)	40.7 ± 5.2	35.2 ± 4.3	36.6 ± 2.6	36.5 ± 5.5	n.s.[b]
Individuals landing on platform (%)	16.3 ± 4.8	38.9 ± 3.9a (****)[d]	43.4 ± 6.8a	64.0 ± 9.7b	**[c]
Individuals landing on upwind half of platform (%)	37.5 ± 26.5 (6)[e] [n.s.][h]	56.3 ± 1.5 (18) [n.s.]	58.2 ± 3.1 (21) [n.s.]	—[f]	n.s.[g]

[a] n.s.: $P > 0.05$; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; ****: $P < 0.0001$.
[b] This row: mean ± SE. Kruskal-Wallis test of differences between pheromone dosages (i.e., clean air not included) (STSC, 1986).
[c] This row: mean ± SD of blocks of 20–25 observations. Different letters indicate significant differences between pheromone treatments (95% LSD intervals).
[d] In parentheses: two-tailed significance level of difference with clean air, t-test (STSC, 1986).
[e] In parentheses (this row): number of landings of which position was recorded.
[f] Not recorded.
[g] As 2, but Mann-Whitney U test.
[h] In brackets: two-tailed significance level of difference from 50% (sign test).

TABLE 6. FLIGHT BEHAVIOR OF *Trichogramma pretiosum* WASPS IN WIND TUNNEL WHEN EXPOSED TO DIFFERENT DOSAGES OF SYNTHETIC SEX PHEROMONE OF *Spodoptera frugiperda*

Parameter	Clean air	Sex pheromone dosage (mg) 0.1	1	3	P^a
Number of observations	200	100	100	100	
Latency to takeoff (sec)	39.3 ± 3.7	52.4 ± 7.6	35.3 ± 5.2	36.7 ± 4.8	n.s.[b]
Individuals landing on platform (%)	14.0 ± 8.6	14.0 ± 7.3a	13.0 ± 5.1a	13.0 ± 2.4a	n.s.[c]
Wasps landing on upwind half of platform (%)	48.5 ± 7.0 (28)[d] [n.s.][e]	44.0 ± 20.7 (14) [n.s.]	48.3 ± 15.7 (13) [n.s.]	20.0 ± 7.3 (13) [n.s.]	n.s.

[a] n.s.: $P > 0.05$; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; ****: $P < 0.0001$.
[b] This row: mean ± SE. Kruskal-Wallis test of differences between pheromone dosages (i.e., clean air not included) (STSC, 1986).
[c] This row: mean ± SD of blocks of 20–25 observations. Different letters indicate significant differences between pheromone treatments (95% LSD intervals).
[d] In parentheses (this row): number of landings of which position was recorded.
[e] In brackets: two-tailed significance level of difference from 50% (sign test).

not affected by odor concentration, and the percentage of wasps that landed on the upwind side did not differ significantly from 50%.

DISCUSSION

The two questions posed at the onset of this study were (1) does the response of *T. pretiosum* to *H. zea* sex pheromone represent a general response to any blend of aliphatic compounds, and (2) could it be a general response due to the artificial environment rather than a host-directed response. Both have been answered negatively. *Trichogramma pretiosum* apparently discriminated between different odors, since it did not respond to the sex pheromone of *S. frugiperda* or to a blend of saturated acetates. This differential response was probably not due to the dosage tested, as we found a clear dose effect with regard to total residence and walking times with *H. zea* and not with *S. frugiperda*. These results correspond with the fact that *H. zea* is a common field host of *T. pretiosum*, while eggs of *S. frugiperda* are very rarely parasitized by this species (Pinto et al., 1986). The fact that *T. pretiosum* does not use fall armyworm sex pheromone as a kairomone seems adaptive: eggs of this moth are difficult to parasitize because they are laid in batches covered by a thick layer of scales (Sparks, 1979) which seem to obstruct the parasitoid (Vickery,

1929; W.J. Lewis, personal observations). The scelionid *Telenomus remus* Nixon does attack *S. frugiperda* in the field (Wojcik et al., 1976). It is more robust than *T. pretiosum* and has been reported to dig successfully through the layer of scales of *Spodoptera litura* (F.) to reach the eggs (Braune, 1982). The same tactic may be used to attack *S. frugiperda*. Alternatively, *T. remus* may be aided by its distally positioned ovipositor (typical for scelionids), which facilitates probing through the layer of scales, while *T. pretiosum* cannot do so because (as in most chalcidoids) its ovipositor is ventrally located (M.R. Strand, personal communication). As in *T. pretiosum*, host acceptance in *T. remus* corresponds with responses to host sex pheromone: it was found to respond to an extract of abdominal tips of *S. frugiperda*, as well as to Z9–14:Ac and Z9–12:Ac, components of its sex pheromone (Nordlund et al., 1983).

Trichogramma spp. have been considered generalists in their choice of hosts (Hase, 1925; Salt, 1934; Sweetman, 1958; Thomson and Stinner, 1989). It has been suggested that plant-generalist natural enemies use more general chemical cues than plant specialists (Vinson, 1976; Sheehan, 1986). By this reasoning one might expect host-generalist parasitoids to use a wider range of chemical cues in their search for hosts than host specialists (Jones, 1986). Our study shows that *T. pretiosum* discriminated between blends of organic chemicals, which—although they differed in several aspects—were all straight-chain even-carbon-numbered aliphatic compounds. Similarly, *T. minutum* responded to a hexane extract of scales of *Choristoneura fumiferana* (Clemens) and not to an extract of *Sitotroga cerealella* (Oliver) scales (Zaborski et al., 1987). Although labeling organisms as generalist or chemical compounds as general seems rather arbitrary, these findings corroborate recent findings that species and/or strains of *Trichogramma* may be much more specific in their search for hosts and their use of semiochemicals than previously thought (van Dijken et al., 1986; Pak, 1988).

From the present results we cannot conclude what caused *T. pretiosum* to discriminate between the three odors. The blends differed in ratio of components, which in turn varied in chain length, presence of double bonds, and functional group. As far as the pheromonal function of these substances is concerned, each of these aspects can confer response specificity (Tumlinson and Teal, 1987). With regard to kairomonal effects, we can only speculate, for instance, that *T. pretiosum* might respond specifically to aldehydes and *T. remus* to acetates. The question of specificity also can be applied to parts of pheromone blends. Is the whole blend essential for a response, or is one component sufficient? In previous olfactometer experiments, *T. pretiosum* responded to the sex pheromone of *H. zea* released by calling moths (Noldus, 1988). However, we do not know which substance was responsible for that response. Noldus and van Lenteren (1985) found that *T. evanescens* did not respond to Z11–16:Ac, the major component of the sex pheromone of *M. brassicae*, in olfactometer

experiments with material tested at a single dosage. The concentration may have been incorrect, or a minor component (or combination of components) may be responsible for *Trichogramma*'s response to the odor of calling female moths. Obviously, additional studies are necessary to answer these questions.

Testing odors against clean air in a wind tunnel may not be the optimal assay for understanding how wasps use chemical cues during foraging in a chemically variable environment. Jones (1986) therefore urges experiments in more natural settings, where the significance of stimuli is studied by subtracting them from a "chemically complete" environment rather than adding them to a "chemically depleted" environment. However, the minute size of *Trichogramma* complicates direct observations of behavior under natural conditions. As a compromise, responses to kairomones can be studied in the laboratory in the presence of plants and other host-related cues. Results of recent experiments following this approach are reported elsewhere (Noldus et al., 1990b).

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Book Review

Behavior-Modifying Chemicals for Insect Management: Applications of Pheromones and Other Attractants. Richard L. Ridgway, Robert M. Silverstein, and May N. Inscoe (eds.). (1990) Marcel Dekker, New York, $195, 761 pp.

Since the discovery, isolation, and identification of the sex attractant pheromone of the silkworm *Bombyx mori*, over 30 years ago, pheromones and parapheromones have been identified for more than 1000 species of insects, ticks, and mites. This plethora of pheromones has spawned innumerable scientific publications and a host of books and review articles on the effect of these powerful chemicals on insect behavior and ways in which this new-found knowledge may possibly be put to use for control of insect pests of crops, orchards, forests, stored commodities, and animals. *Behavior-Modifying Chemicals for Insect Management: Applications of Pheromones and Other Attractants* presents a comprehensive international viewpoint of the present status of pheromones relative to achievements in this area and future prospects for pheromones becoming a significant component in pest management schemes worldwide.

The book comprises 39 chapters subdivided into seven parts. The first part covers the history and science of pheromones relative to chemical analysis, identification, and synthesis; formulations; and principles governing control strategies such as monitoring, mass trapping, and mating disruption. Parts 2–5 present specific case histories of successful applications as control agents in horticultural crops (Part 2), forest situations (Part 3), and field crops (Part 4). Part 5 highlights the use of pheromones as tools for monitoring insect pests of stored commodities and dipterous animal pests, specifically tsetse flies and house flies.

Part 6 presents the commercial side of pheromone technology, especially the requirements for registration and the regulations governing pheromone usage in the United States, Japan, Israel, Australia, and various European countries. This section is summed up nicely by C.A. O'Connor (Chapter 36) who states "Because of the time and cost required for pheromone registration, pheromones will probably be developed for use on only major crops or in conjunction with conventional pesticides." It appears that many countries are watching closely the development of events surrounding the registration and regulation of pheromones in the United States. A more relaxed attitude on the part of the U.S. Environmental Protection Agency in getting pheromones approved and into the

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marketplace most probably will be echoed by regulatory agencies in other countries.

The concluding chapter of Part 6 provides an alphabetical listing of 257 arthropods for which pheromones and other behavioral chemicals were commercially available in 1988. The listings are cross-referenced by scientific and common names and include the chemically active compounds as reported by the different suppliers. A partial list of suppliers of insect pheromones, synthetic attractants, and traps is provided in the appendix. A listing of the pheromones actually registered by EPA through 1987 are to be found in Chapter 35.

The 39th and final chapter (Part 7) is appropriately titled "Pheromones: Prophecies, Economics, and the Ground Swell." Considering all of the insect sex attractant pheromones that have been identified, the number of cases in which these compounds actually have been used to effect a degree of pest control are disappointingly low. The reasons for this are many and varied, and it is easy to single out a few, such as overregulation by government and underfunding and short-term profit motives on the part of industry. The truth is that economics still rules the day. Economics may become less the issue, of course, as society's concern for the environment grows. As Arn noted "A concerted effort by industry and research scientists (and I might add government) will be needed if pheromones are to find a permanent place in the toolbox of integrated control."

True to its intent, the 761-page book is packed with up-to-date information on all aspects on the use—a few real but most in the realm of "potential"—of pheromones and behavior-modifying chemicals for insect pest management. The authors are commended for their concise and crisp job of writing and the editors for their superb editing and layout, providing for a smooth, logical flow of the book. The figures are sharp and legible, not too cute and overdone.

The book is a must for all who work with insect sex attractant pheromones and other behavior-modifying compounds, including those whose interest in pheromone research may be purely academic. Someday they may have to justify their intellectual pursuits to uninformed administrators, in which case the information gathered here would be invaluable. The biggest drawback to the book is its price tag, $195 per copy. If individual pockets are not deep enough, then interested parties should make sure that their library or department secures a reference copy.

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Letter to the Editor

Ecological Implications of the Destruction of Juglone (5-hydroxy-1,4-naphthoquinone) by Soil Bacteria

There have been two recent reports of the isolation of soil bacteria with the metabolic capability to destroy juglone (Rettenmaier et al., 1983; Schmidt, 1988). Rettenmaier et al. (1983) isolated an organism from German soil that could convert juglone to 2-hydroxymuconic acid. I later isolated a similar bacterium from soil beneath black walnut (*Juglans nigra* L.) trees in the United States. This bacterium could grow rapidly using juglone as its sole source of carbon and energy (Schmidt, 1988). In my paper I also argued that, given the susceptibility of juglone to destructive biotic and abiotic processes in soil, it seemed unlikely that juglone could persist at phytotoxic levels in the field. Recently, Williamson and Weidenhamer (1990) critiqued my conclusions about the fate of juglone. I found their analysis to be thought-provoking enough to warrant a reply. Although I agree that neither the arguments for nor against allelopathy are free of ambiguity, there is still no evidence, that I have seen, that juglone persists at phytotoxic levels or acts as a phytotoxin in the field.

Williamson and Weidenhamer (1990) claim that two studies (Ponder and Tadros, 1985; Weidenhamer et al., 1989) present evidence that juglone can persist at phytotoxic levels in the field. In the first of these studies, Ponder and Tadros (1985) took the laudable step of measuring juglone levels in soils at varying distances from black walnut trees. One problem with this work was that they used chloroform to extract juglone from the soil. Chloroform is a strong organic solvent that can extract juglone from litter, soil organic matter, and even the membranes of microorganisms that may be removing juglone from the soil solution. As pointed out by Kaminsky (1980, 1981), it is the concentration of toxins available to plant roots in the soil solution that is important in studies of allelopathy, because it is this available pool that can be taken up by plants. In a recent study, De Scisciolo et al. (1990) found that after only 10 min of incubation, less than 1% of the juglone added to soil at a concentration of 200 μg/g could be recovered when water was used as the solvent rather than chloroform. Thus, the study of Ponder and Tadros (1985) cannot be used as evidence that juglone concentrations in the field are at high enough levels to inhibit plant growth. Indeed, Ponder and Tadros (1985) even noted that "the actual concentrations of juglone that the European black alder and autumn olive are

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exposed to in the soil solution are probably less than the amounts found in our analysis because of juglone's high solubility in chloroform."

The study of Weidenhamer et al. (1989) was designed to examine the interactions between resource competition and allelopathy. To do this, they grew tomato plants in soil from beneath and away from black walnut trees. Some of their results indicated that there may be something in the soil beneath black walnut trees that inhibited plant growth. However, other data that they presented indicated stimulatory effects on the tomato plants. For instance, in all six experiments described, there were more tomato plants surviving in the soil from beneath black walnut trees than in the "control" soils, despite the fact that equal densities were sown in both soils. This difference in established densities also makes any comparison of the two soils questionable for assessing the effects of plant density on allelopathy. In addition, in this study there was no control for soil nutrient availability; that is, the two soils used were not shown to be similar in nutrient content. In fact, no characterization of the soils was given. It is well documented that physical and chemical properties of soil can vary dramatically as a function of the distance away from the bases of individual trees (Rhia et al., 1986; De Scisciolo et al., 1990). Finally, Weidenhamer et al. (1989) did not quantify juglone in any of the soils they studied. Thus, even if the data did indicate an allelopathic effect, no evidence was presented to show that juglone was the responsible chemical.

Reports of the adverse effects of walnut trees on surrounding vegetation date back several thousands of years (Willis, 1985), but there is still no convincing evidence that juglone is the cause (De Scisciolo et al., 1990). To determine if juglone is important in interactions between plants, tightly controlled experiments are needed that demonstrate the presence of juglone in a form available to plants, combined with quantitative evidence that juglone affects plant distribution in the field. Ideas as to how such studies should be performed already exist in the literature (De Scisciolo et al., 1990; Fuerst and Putnam, 1983; Schmidt and Reeves, 1989; Stowe, 1979; Willis, 1985).

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